2010

Genetic regulation of bovine milk fatty acid composition: Improving the healthfulness of milk through selection

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Genetic regulation of bovine milk fatty acid composition: Improving the healthfulness of milk through selection

by

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A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Co-majors: Biochemistry;
Nutritional Sciences (Molecular and Cellular Nutrition)

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Ames, Iowa

2010

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ABSTRACT

The current study was designed to identity polymorphisms in the genes involved in milk lipid biosynthesis to provide animal breeders with tools that allow selection of animals producing milk with healthier fatty acid composition. High concentrations of saturated fatty acids (SFA) in human diets are known to increase plasma cholesterol concentrations and, as a result, increase the risk of developing cardiovascular diseases (CVD), the number one cause of death worldwide. Because bovine milk is one of the primary sources of SFA and individual atherogenic fatty acids such as palmitic (16:0) and myristic (14:0) in human diets the improvement of the healthfulness of milk through selection becomes one of the primary measures that has been taken with the intention of decreasing the incidence of CVD among humans.

The candidate gene approach was used to address the objectives of the study. Genes involved in milk triacylglycerol (TAG) biosynthesis, fatty acid uptake into mammary gland and fatty acid transport inside the mammary epithelial cells, and transcriptional regulation of some lipogenic genes were investigated. DNA sequencing was used to discover single nucleotide polymorphisms (SNPs) in the genes of interest. After genotyping animals on the study for the discovered SNPs, the intragenic haplotypes were reconstructed and tested for the association with milk fatty acid composition.

The glycerol-3-phosphate acyltransferases-1 and -4 (GPAT1 and GPAT4), 1-acylglycerol-3-phosphate acyltransferase-1 (AGPAT1), and phosphatidate phosphatase (LPIN1) genes from the TAG biosynthetic pathway were studied in the first set of experiments to test the associations of the polymorphisms in those genes with milk fatty acid

composition. The polymorphisms in GPAT4 were associated with large differences in atherogenic index (AI), concentrations of SFA, unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), SFA/UFA, concentrations of capric (10:0), lauric (12:0), palmitic (16:0), and oleic ($18:1^{c9}$) acids, CLA ($18:2^{c9, t11}$), C16 and C18 desaturation indices in milk. The size of the effects of GPAT4 polymorphisms for some of the traits was numerically at least the same or larger compared with the effect of DGAT1 A232K mutation, making the polymorphisms in GPAAT4 to be a very valuable tool for the improvement of the healthfulness of milk. Other polymorphisms significantly associated with the studied traits in the first set of experiments were in GPAT1 for milk fat percentage, concentrations of short- and medium-chain SFA, and myristoleic ($14:1^{c9}$) acid concentration, and in AGPAT1 for the concentrations of linoleic ($18:2^{c9, c12}$) acid and other UFA. The polymorphisms in GPAT1 can be used to select for animals producing milk with higher percentage of fat and desirable concentrations of short- and medium-chain SFA. The polymorphisms in AGPAT1 can be used to select for animals producing milk with higher concentration of UFA and linoleic ($18:2^{c9, c12}$) acid, in particular.

In the second set of experiments, the polymorphisms in the solute carrier family 27 (SLC27A6), isoform A6 and fatty acid binding proteins-3 and -4 (FABP3 and FABP4) genes involved in fatty acid uptake into mammary gland and fatty acid transport inside the mammary epithelial cells were tested for the association with milk fatty acid composition. The haplotype effects of SLC27A6 were associated significantly with milk fat percentage, AI, the concentrations of SFA, UFA, MUFA, SFA/UFA, the concentrations of capric (10:0), lauric (12:0), myristic (14:0), and palmitic (16:0) acids. The size of the haplotype effects of SLC27A6 on the studied traits was large and numerically similar to the size of allelic effects

of DGAT1 A232K mutation that makes the polymorphisms in SLC27A6 as valuable as the of DGAT1 A232K mutation to select for animals producing milk with higher fat percentage and healthier fatty acid composition. The haplotype effects of FABP4 were associated significantly with the concentrations of SFA, UFA, MUFA, PUFA, SFA/UFA, the concentrations of linoleic ($18:2^{c9, c12}$) acid, CLA ($18:2^{c9, t11}$), and C18 desaturation index.

The sterol regulatory element binding protein-1c (SREBP-1c) is involved in the transcriptional regulation of lipogenesis and its proteolytic activation is controlled by SREBP cleavage-activating protein (SCAP) and insulin-induced genes (Insig) that are all part of the SREBP pathway. In the third set of experiments, the significant association of the overall haplotype effect of SREBP1 with the concentrations of myristic (14:0), myristoleic ($14:1^{c9}$) acids, and C14 desaturation index were detected. The overall haplotype effect of Insig1 was associated with the concentrations of PUFA and linoleic ($18:2^{c9, c12}$) acid. There were no significant associations with milk fatty acid composition determined for SCAP.

In conclusion, we were able to identify polymorphisms in a number of genes that were associated significantly with milk fat percentage and fatty acid composition. The information about those polymorphisms can be used to select for animals producing healthier milk.

GENERAL INTRODUCTION

Dissertation Organization

The research described here is presented in the form of three publications prepared for the submission to scientific journals as a partial fulfillment of the requirements for the Doctor of Philosophy degree. Each paper is complete in itself and includes an abstract, introduction, materials and methods, results, discussion, conclusions, and references. The title of the first paper, prepared for the submission to BMC Genomics, is "Effects of the polymorphisms in GPAT1, GPAT4, AGPAT1, and LPIN1 genes from the triacylglycerol biosynthetic pathway on milk fatty acid composition in dairy cattle". The second paper, prepared for the submission to Physiological Genomics, entitled "Effects of the polymorphisms in FABP3, FABP4, and SLC27A6 genes on bovine milk fatty acid composition". The third paper, entitled "Effects of genetic polymorphisms in the SREBP pathway on milk fatty acid composition in dairy cattle", is prepared for the submission to Animal Genetics. The manuscripts are formatted according to the requirements of each journal. The three papers are preceded by a literature review and followed by general conclusions containing a general discussion, future research, and references sections. The appendix with results not included into the publications concludes the dissertation.

Literature Review

Cardiovascular disease occurrence

Cardiovascular diseases (CVD) are a group of diseases (International Classification of Diseases 10, 100-199; Q20-Q28) that includes high blood pressure, coronary heart disease, heart failure, and stroke. The importance of studying the etiology of CVD and developing the preventatives and treatments for CVD comes from the fact that, according to the World Health Organization, CVD are the number one cause of death globally with an estimated 17.1 million deaths from CVD in 2004, representing 29% of all global deaths from diseases. Low- and middle-income countries are affected the most, with 82% of total CVD deaths occurring in those countries. It is projected that in 2030 almost 23.6 million people worldwide will die from CVD.

As of the year of 2009, 81,100,000 American adults (more than 1 in 3) have at least one type of CVD. More precisely, 74,500,000 suffer from high blood pressure (hypertension), 17,600,000 have coronary heart disease, 5,800,000 had heart failure, and 6,400,000 suffered from stroke [1]. Mortality data show that CVD, as the underlying cause of death, accounted for 34.3% (831,272) of all deaths in the United States in 2006, with 33% of all CVD deaths occurring before the age of 75 years [1]. Nearly 2,300 Americans die of CVD each day, an average of one death every 38 seconds. The CVD claims more lives each year than cancer, chronic lower respiratory diseases, and accidents combined. The probability at birth of eventually dying of major CVD is 47%, whereas the chance of dying of cancer is 22%. Other probabilities are 3% for accidents, 2% for diabetes mellitus, and 0.7% for HIV.

In countries other than the United States, the death rates per 100,000 people per year from CVD calculated separately for men and women in the age group between 35 and 74 years were the following: Russian Federation (2002) 3,186.9 and 1,192.4; Germany (2006) 788.5 and 402.4; Mexico (2001) 1,055.8 and 713.2; Canada (2004) 705.3 and 432.7; and France (2006) 793.6 and 358.4 [1].

Fatty acids and human plasma cholesterol concentrations

Dietary SFA as a class can raise plasma cholesterol, LDL-cholesterol, and HDL-cholesterol concentrations of humans. According to a meta-analysis based prediction model that explained 74% of the variability related to the changes in plasma cholesterol concentrations, a 1% increase in dietary total energy from SFA will result in 1.9 mg/dL increase in plasma total cholesterol concentration [2]. A similar prediction model that explained 65% of the variability in plasma LDL-cholesterol concentrations indicated a 1.8 mg/dL rise in plasma LDL-cholesterol concentration for every 1% increase in dietary total energy from SFA [2]. A weaker prediction model that explained only 41% of the variability in HDL-cholesterol concentrations indicated a 0.3 mg/dL increase in plasma HDL-cholesterol concentration for every 1% increase in dietary total energy from SFA [2]. Similar results about the effects of SFA on plasma cholesterol concentrations were obtained in other studies [3-9].

Separate SFA, however, do not have the same effects on plasma cholesterol concentrations. Specifically, palmitic (16:0) and myristic (14:0) acids raise plasma total and LDL-cholesterol concentrations much higher compared with other SFA [4]. Palmitic acid (16:0) is the major SFA in human diets. It is predominantly found in milk and other dairy products, beef tallow and lard, and some vegetable oils. Myristic acid (14:0) in human diets

comes mainly from milk and other dairy products and some vegetable oils. The ability of myristic acid (14:0) to raise plasma cholesterol concentrations is at least the same as palmitic acid (16:0) or even higher [4]. The percentage of myristic acid (14:0) in human diets is relatively low and therefore its hypercholesterogenic effects are not considered to be as severe as those caused by the presence of palmitic acid in the diet [3].

Lauric acid (12:0) is another SFA considered to be hypercholesterolemic [10]. Some data, however, show that lauric acid (12:0) is only mildly hypercholesterolemic [11]. A meta-analysis based summary of recent studies shows that lauric acid indeed can increase plasma total cholesterol and LDL-cholesterol concentrations, but it decreased total cholesterol to HDL-cholesterol ratio [12].

Stearic (18:0) acid and short- and medium-chain SFA have little or no effect on blood total and LDL-cholesterol concentrations [7, 12-17]. The fact that short- and medium-chain fatty acids are absorbed directly into portal circulation and not into the blood stream via chylomicrons in the lymphatic system might explain their neutral effects on blood cholesterol concentrations [3]. The hypocholesterolemic effect of stearic acid (18:0) can be explained by its rapid desaturation to oleic acid ($18:1^{c9}$) as compared with palmitic acid (16:0) in which desaturation to oleic acid ($18:1^{c9}$) first will require an elongation step [18].

In major foods, the MUFA are represented mainly by oleic acid ($18:1^{c9}$). The effects of MUFA on plasma cholesterol concentrations are considered to be neutral or mildly hypocholesterolemic [4, 5, 19]. Nevertheless, a high intake of MUFA may increase risk of developing CVD [20]. The effects of PUFA on plasma cholesterol concentrations, however, are hypocholesterolemic. According to a meta-analysis based prediction model that explains 74% of variability associated with differences in plasma cholesterol concentrations, a 1%

increase in dietary total energy coming from PUFA will result in 0.99 mg/dL decrease in plasma total cholesterol concentration [2].

The PUFA are classified into n-3 and n-6 fatty acids found primarily in fish and different vegetable oils, respectively [8]. Linoleic acid ($18:2^{c9, c12}$, n-6) that is the major n-6 fatty acid in human diets was shown to decrease CVD risks by promoting a desirable profile of lipoprotein fractions and TAG concentration in blood [12, 21, 22]. Prospective epidemiological studies confirm cardioprotective role of linoleic acid ($18:2^{c9, c12}$, n-6) by showing that its consumption decreases CVD risks [23]. A negative view about dietary linoleic acid ($18:2^{c9, c12}$, n-6), however, was developed because it can be metabolized through a few elongation and desaturation steps to form arachidonic acid ($20:4^{c5, c8, c11, c14}$, n-6), which can be metabolized further to prostaglandins and leukotrienes known to be prothrombotic, proinflammatory, and vasoconstricting [24]. Discovery of a possible competition of n-6 with n-3 PUFA for cyclooxygenase that can lead to a decreased production of antiinflammatory prostaglandins from n-3 PUFA added more support to a negative view about n-6 PUFA and linoleic acid ($18:2^{c9, c12}$, n-6) in particular. The importance of considering the n-6 PUFA to n-3 PUFA ratio of 6:1 was suggested, then, to maintain increased production of antiinflammatory prostaglandins [22, 25]. Nevertheless, linoleic acid ($18:2^{c9, c12}$, n-6) is still the most effective PUFA for lowering blood cholesterol concentrations and decreasing risk of CVD [26].

The contributions of individual fatty acids to atherogenic potential for a lipid source or a diet are summarized by an atherogenic index (AI) that was developed by Ulbricht and Southgate in 1991 [27]. The AI, described by these authors, ranks mixtures of fatty acids according to their propensity to cause atherogenesis, as predicted from concentrations of

individual fatty acids in the dietary lipid. The AI is calculated as: (12:0 + 4 × (14:0) + 16:0) ÷ (ΣMUFA + ΣPUFA).

Trans fatty acids from partially hydrogenated vegetable oils are well known to increase the risk of CVD in humans [28]. The effects of trans fatty acids of animal origin, however, are quite opposite [29, 30]. The predominant trans fatty acid in milk is vaccenic acid ($18:1^{t11}$) that can be converted by delta-9 desaturase to conjugated linoleic acid (CLA; $18:2^{c9, t11}$) known to have several potential health benefits. Feeding CLA to rabbits decreased plasma total and LDL-cholesterol, the LDL-cholesterol to HDL-cholesterol ratio, and TAG concentration [31]. Similar effects of CLA on plasma cholesterol metabolites were observed in a hamster study [32]. Another hamster study showed that butter naturally enriched with vaccenic acid and CLA decreased plasma total cholesterol and LDL-cholesterol concentrations in treated animals compared with the control [33]. It was also shown that not all CLA isomers have the same effect with respect to plasma cholesterol concentrations. For example, trans-10, cis-12 CLA ($18:2^{t10, c12}$) was shown to modify HDL particles in a way that makes them more atherosclerosis-promoting [34].

In humans, intake of dairy products naturally enriched in CLA was shown to increase CLA concentration in plasma and cellular lipids [35]. The health-promoting effects of CLA on blood lipid profiles in humans are quite inconsistent, though. For example, some researchers observed improvement in cholesterol-containing lipoprotein profile in blood [36] whereas others did not [37, 38]. The one clear observation is that different CLA isomers have different effects on plasma lipid concentrations, with trans-10, cis-12 CLA being detrimental in terms of plasma cholesterol concentrations [36]. A possible role for CLA as a health-promoting compound awaits future discovery.

Fatty acids also are known to affect insulin secretion. Studies in rats showed that different dietary fatty acids influenced insulin secretion to a different extent, with caprylic (8:0), linoleic ($18:2^{c9, c12}$), oleic ($18:1^{c9}$), palmitic (16:0), and stearic (18:0) acids causing an increase in insulin secretion by 3.4, 5.3, 9.4, 16.2, and 21.0 fold, respectively [39]. All together, the results of these studies suggest that fatty acid composition of a diet can affect insulin secretion. High-fat diets, however, are known to induce insulin resistance accompanied by increased TAG deposition in liver and skeletal muscle. The developmental mechanism of insulin resistance is still unknown, but certain fatty acids acting through the novel protein kinase C family members are thought to be responsible for the effect [40, 41]. Palmitic acid (16:0), in particular, was responsible for developing insulin resistance in hypothalamus and skeletal muscle [42, 43]. Fatty acids also are thought to increase the risk of some diseases other than CVD, but the absence of good biomarkers for those diseases makes it difficult to study the effects of fatty acids on them.

Environmental factors affecting milk fatty acid composition in dairy cattle

The major environmental factors that are known to affect milk fatty acid composition are diet, stage of lactation, and season of the year. Nutrition can affect lipid concentration and fatty acid composition in milk, but the extent to which the change can occur differs among species and between different stages of lactation for the same species [44]. In nonruminants, for example, milk fatty acid composition reflects fatty acid composition of their diets. In ruminants, however, the majority of dietary unsaturated fatty acids are reduced by biohydrogenation in the rumen and many new fatty acids of bacterial origin are introduced into the milk, which makes it difficult to control fatty acid composition of milk by dietary

means. At the same time, diet can affect the microbial population of the rumen and as a consequence alter milk fatty acid composition.

It is important to know the contribution of de novo lipogenesis in the mammary gland to milk lipid biosynthesis to better understand the effects of a diet on milk fatty acid composition. In animals such as elephants who derive more than 90% of their fatty acids in milk from de novo lipogenesis, milk contains mainly short-chain and medium-chain fatty acids [44]. In seals, however, who rely exclusively on their adipose tissue depots to provide fatty acids for milk TAG biosynthesis, the majority of milk fatty acids are long-chain fatty acids. In ruminants, when they are not in a negative energy balance state, de novo lipogenesis contributes only 50% of all fatty acids in milk and as a consequence milk from ruminants contains many groups of fatty acids [45]. Nonruminants utilize glucose as a major carbon source for de novo lipogenesis, whereas ruminants utilize acetate produced from carbohydrate fermentation in the rumen along with β-hydroxybutyrate, another product of fermentation, providing about one half of the first four carbons of de novo synthesized fatty acids [46].

In dairy cattle, fatty acids taken up from blood by the mammary gland are derived from NEFA and very-low density lipoproteins of intestinal origin [47]. The blood NEFA comes mainly from lipolysis in adipose tissue that accounts for less than 10% of fatty acids used for milk lipid biosynthesis unless cows are in negative energy balance when lipolysis in adipose tissue provides the majority of fatty acids for milk lipid biosynthesis [48, 49]. Other fatty acids are derived from the diet.

Earlier attempts to study the effect of a diet on milk fat yield and fatty acid composition were directed towards addressing the milk fat depression (MFD) problem. It

was discovered that diets containing large proportion of readily digestible carbohydrates and low concentration of effective digestible fiber can cause MFD [49, 50]. Another major contributor to MFD is the addition of dietary supplements containing plant and fish oils rich in PUFA [51]. A number of theories were proposed to explain MFD, with one of the first theories linking changes in the concentrations of volatile fatty acids (VFA) in the rumen to the decrease in milk fat yield for a variety of diets [49]. It was proposed, first, that a decrease in the production of acetate and butyrate in the rumen is responsible for limiting milk lipid biosynthesis in the mammary gland [50]. A variation in milk lipid concentration estimated to be up to 80% was associated with changes in molar proportions of VFA in the rumen [44]. Other researchers, however, do not fully support the MFD theory based on the differences in acetate and butyrate production in the rumen, because there is some evidence showing that a decrease in ruminal acetate molar proportion is a result of increased propionate production rather than an actual decrease in acetate production [44].

Another theory on MFD related an increase in plasma insulin concentrations in response to low fiber high carbohydrate diets with the repartitioning of nutrients from lipid biosynthesis in mammary gland towards lipid biosynthesis in adipose tissue [52]. The weakness of this theory comes from the fact that a number of studies looking at the effects of insulin on nutrient repartitioning were conducted on cows in a negative energy balance state when lipolysis in adipose tissue, liable to be inhibited by increased plasma insulin concentration, was the main source of fatty acids for lipid biosynthesis in mammary gland [44]. Under conditions of positive energy balance, infusions of insulin in dairy cows were not able to cause repartitioning of nutrients towards lipid biosynthesis in adipose tissue [49].

The final theory about MFD called “trans fatty acid theory” was proposed to indicate the effect of diet on PUFA biohydrogenation in the rumen [49]. It was proven that feeding dairy cows low-fiber high-carbohydrate diet leads to the production of trans-10 octadecenoic acid ($18:1^{t10}$) rather than trans-11 octadecenoic acid ($18:1^{t11}$) as an intermediate of ruminal biohydrogenation of dietary PUFA [51]. Then, it was hypothesized that trans-10 octadecenoic acid ($18:1^{t10}$) was responsible for the inhibition of milk lipid biosynthesis. The increased concentration of trans-10 octadecenoic acid ($18:1^{t10}$) in milk from cows fed diets that typically cause MFD was another indication of potential involvement of the trans isomer in MFD [53].

Later studies that focused on developing ways to increase CLA concentration in milk serendipitously discovered that CLA ($18:2^{t10, c12}$) is a more potent inhibitor of milk lipid biosynthesis than is trans-10 octadecenoic acid ($18:1^{t10}$) [54]. That discovery prompted the introduction of a new MFD theory called “biohydrogenation theory” that was a modification of the earlier proposed “trans fatty acid theory” [44]. The knowledge gap about the mechanism of MFD was closed when it was shown that CLA ($18:2^{t10, c12}$) not only inhibits milk lipid synthesis in mammary gland but also repartitions nutrients towards lipid biosynthesis in adipose tissue [55].

Lipid supplementation of dairy cow rations in the form of oilseeds and oils rich in linoleic ($18:2^{c9, c12}$) and linolenic ($18:3^{c9, c12, c15}$) acids decreased concentrations of palmitic acid (16:0) and short- and medium-chain fatty acids (C6 to C14) in milk at the expense of increasing stearic (18:0) acid concentration [56]. Such a change in milk fatty acid concentrations can be explained by increased uptake of dietary fatty acids, mainly stearic (18:0) acid, by the mammary gland that causes an inhibition of de novo lipogenesis [57]. The

concentrations of odd-chain fatty acids in milk also were decreased with the dietary lipid supplementation, supporting the argument about their origin in favor of de novo biosynthesis in the mammary gland rather than in the rumen [56].

In addition to increased concentration of stearic acid (18:0) in milk, dietary supplementation with linoleic ($18:2^{c9, c12}$) and linolenic ($18:3^{c9, c12, c15}$) acids also increased the concentration of vaccenic acid ($18:1^{t11}$) and other cis- and trans-18:1 isomers [56]. The recovery of linoleic ($18:2^{c9, c12}$) and linolenic ($18:3^{c9, c12, c15}$) acids in milk after their dietary supplementation was minimal because of a high percentage of their biohydrogenation in the rumen unless those fatty acids were protected from the reducing environment of the rumen [56, 58, 59].

Stage of lactation affects milk fatty acid composition, because it determines whether cows use adipose tissue or both adipose tissue and de novo lipogenesis in the mammary gland to provide fatty acids for milk TAG biosynthesis. At the beginning of lactation, cows usually are in negative energy balance, relying more extensively on lipolysis in adipose tissue to provide fatty acids for milk lipid biosynthesis [57]. This situation results in the concentration of short- and medium-chain fatty acids (C_6 to C_{14}) in milk being low at the beginning of the lactation because of the inhibitory effects of mobilized long-chain fatty acids on de novo lipogenesis in mammary tissue. With the progression of lactation, the concentration of short- and medium-chain fatty acids in milk gradually increases. There were no obvious seasonal effects on milk fatty acid composition, but the trend was to have lower concentration of short- and medium-chain fatty acids during warm months of the year probably because of the higher dietary fat intake at that time [57].

Genetic regulation of milk fatty acid composition in dairy cattle

Heritability of milk fatty acids

The heritability values for individual milk fatty acids range from 0.00 to 0.50 and higher [60-65]. Such a variation in heritability values can be explained by differences in units used to express milk fatty acid concentrations, methods used to measure milk fatty acid concentrations, models used to analyze the data, and number of samples available for the analysis. When milk fatty acid concentrations were expressed as g/100 g of milk total lipids, the heritability values for the major milk fatty acids were the following: 0.07-0.19 for myristic acid (14:0), 0.03-0.20 for palmitic acid (16:0), 0.08-0.28 for stearic acid (18:0), 0.06-0.17 for oleic acid ($18:1^{c9}$), 0.14 for SFA, and 0.14-0.24 for MUFA [60-62, 64]. The heritability values for milk fatty acids were 0-0.49 for myristic acid (14:0), 0.09-0.31 for palmitic acid (16:0), 0.19-0.24 for stearic acid (18:0), 0.06-0.18 for oleic acid ($18:1^{c9}$), 0.05 for SFA, and 0.08 for MUFA when fatty acid concentrations were expressed as weights as a proportion of total fat fatty acid weight [63, 65]. The heritability values of milk fatty acids are generally higher than above values when fatty acid concentrations are expressed per volume of milk [64].

The genetic correlation between palmitic acid (16:0) and the percentage of milk fat was high and positive (0.65-0.74) [62, 65], implying that selection for high milk fat percentage will result in increased concentration of palmitic acid (16:0) in milk. The concentration of MUFA with 18 carbon atoms, however, had a negative genetic correlation with the percentage of milk fat (from -0.55 to -.074) [62, 65]. Other fatty acids that had a positive genetic correlation with milk fat percentages were lauric (12:0) and stearic (18:0) acids with values for the correlation coefficients as 0.55 and 0.84, respectively [60].

QTL affecting milk lipids

One of the earlier studies that mapped quantitative trait loci (QTL) controlling milk production in dairy cattle using a granddaughter design identified QTL on bovine chromosomes 1, 9, and 10 that were associated with milk fat yield and QTL on bovine chromosomes 6 and 20 that were associated with milk fat percentage [66]. Another study mapped QTL for milk fat percentage to bovine chromosomes 6, 14, 20, and 26 and QTL for milk fat yield to bovine chromosomes 9 and 14, confirming some results from earlier studies and identifying new QTL that were not reported before [67]. Further studies mapped QTL for milk fat percentage to bovine chromosomes 3, 6, and 14 [68], QTL for milk fat yield to bovine chromosomes 19 and 26, and milk fat content to bovine chromosome 19 [69], QTL for milk fat percentage to bovine chromosome 3, and QTL for milk fat yield to bovine chromosome 26 [70]. The findings from numerous studies looking at QTL effects on milk production traits in dairy cattle were summarized in the form of QTL maps [71, 72].

Discoveries of numerous QTL for milk production traits in dairy cattle prompted researchers to identify causal genes for the QTL of interest. The QTL for milk fat percentage and yield located on bovine chromosome 14 was mapped more precisely [73-75] because of the highly significant effects, leading to the discovery of a nonconservative substitution of lysine by alanine at 232 position in the DGAT1 protein [76]. Since then, numerous publications characterized the K232A mutation in DGAT1 gene in relation to milk fat yield, percentage, and milk fatty acid composition [77-84]. Recent studies, however, pointed out that the DGAT1 K232A mutation is not solely responsible for the milk production QTL on bovine chromosome 14 [85]. Moreover, alleles of the DGAT1 promoter derived from the

variable number of tandem repeat polymorphisms were associated with milk fat percentage in animals that were homozygous for the allele 232A in DGAT1 [86]. The presence of additional genetic polymorphisms in DGAT1 and elsewhere on bovine chromosome 14 indicates that the DGAT1 K232A polymorphism cannot completely explain the variability in milk production traits attributed to the QTL on the same chromosome, and thus new polymorphisms are awaiting their discoveries.

The discovery of significant QTL for milk production traits [66] and the presence of casein genes on bovine chromosome 6 drew attention of many researchers to try to fine-map the QTL and identify the candidate genes. Earlier studies identified QTL for milk fat yield mapped near casein genes on bovine chromosome 6 [66, 87]. Another study identified a second QTL for milk fat yield on bovine chromosome 6 [88] that was fine-mapped [89, 90], and peroxisome proliferator-activated receptor-γ coactivator-1α (PPARGC1A) was identified as a functional candidate gene for the discovered QTL [91]. A significant association between a SNP in intron 9 of PPARGC1A gene and milk fat yield was found [91], but it was not confirmed in a later study [92].

Single nucleotide polymorphisms associated with milk fatty acid composition

Following studies designed to identify QTL in dairy cattle in relation to milk production and quality, a discovery of a polymorphism in DGAT1 gene that is associated with milk fat yield was the first example showing an association between genetic polymorphism in the form of single nucleotide mutations and milk fat yield [76, 93]. Since then, it was shown that DGAT1 polymorphism affects not only milk fat yield but also milk fatty acid composition [82, 84, 94]. The DGAT1 gene is located on bovine chromosome 14 and encodes an enzyme that

catalyzes the rate-limiting step in TAG biosynthesis by adding acyl-CoA to sn-3 position on the glycerol backbone. Following the discovery of DGAT1 gene location [95], it was demonstrated that a knock-out mice for DGAT1 gene had impaired lactation [2], prompting researchers to look at the effects of mutations in DGAT1 gene on milk fat synthesis. A nonconservative substitution of lysine by alanine (K232A) in DGAT1 was identified to be responsible for the effects of that enzyme on milk fat yield, with the lysine-encoding allele being associated with higher milk fat yield [76, 93]. A functional conformation of the K232A mutation in DGAT1 was made later by showing that the K allele was characterized by higher V_{max} for the DGAT1-catalyzed reaction compared with the A allele, which explained why animals with the K allele had higher milk fat yield [96].

The DGAT1 232K polymorphism is associated with a higher percentage of palmitic acid (16:0), smaller percentages of myristic (14:0) acid, MUFA with 18 carbon atoms, and CLA ($18:2^{c9, t11}$), and higher SFA/ unsaturated fatty acid (UFA) ratio [82]. Fatty acid desaturation indices defined as the ratio of the percentage of MUFA to the sum of the percentages of MUFA and SFA with the same number of carbon atoms expressed on a percentage bases were studied for milk fatty acids with respect to DGAT1 K232A polymorphism [84]. The DGAT1 allele was associated with smaller desaturation indices for capric (10:0), lauric (12:0), myristic (14:0), and palmitic (16:0) acids with higher desaturation indices for stearic (18:0) and vaccenic ($18:1^{t11}$) acids, and with higher total desaturation index. The observed differences in milk fatty acid composition for DGAT1 K232A polymorphism also can be attributed to the fact that DGAT1 has a preference for adding short-chain and UFA to the sn-3 position of TAGs [97].

After the discovery of SNPs in stearoyl-CoA desaturase 1 (SCD1) gene that were associated with beef fatty acid composition in Japanese Black cattle [98], there were a few studies describing the effects of the same SNPs on milk fatty acid composition in dairy cattle, but the results of those studies were slightly inconsistent [84, 99, 100]. The SCD1 gene located on bovine chromosome 26 encodes an enzyme that catalyzes the desaturation of long-chain SFA, primarily palmitic (16:0) and stearic (18:0) acids, to corresponding MUFA at delta-9 carbon atom. A nonconservative substitution of valine by alanine (T878C) in exon 5 of the SCD1 gene was associated with greater concentration of MUFA in beef from Japanese Black cattle [98]. The T878C SNP in dairy cattle in one study was associated with higher concentrations of caproleic ($10:1^{c4}$) and myristoleic ($14:1^{c9}$) acids for C allele (alanine) [99], and in another study was associated with higher concentrations of MUFA, myristoleic ($14:1^{c9}$), and oleic ($18:1^{c9}$) acids for the same C allele [100]. Yet another study reported that the C allele (alanine) was associated with lower concentrations of capric (10:0), lauric (12:0), myristic (14:0), and palmitoleic ($16:1^{c9}$) acids and of CLA ($18:2^{c9, t11}$) and higher concentrations of caproleic ($10:1^{c4}$), lauroleic ($12:1^{c4}$), myristoleic ($14:1^{c9}$), stearic (18:0), and vaccenic ($18:1^{t11}$) acids [84].

The fatty acid synthase (FAS) gene located on bovine chromosome 19 was the focus of recent studies designed to identify SNPs associated with differences in milk fatty acid composition and milk fat yield [92, 101, 102]. Earlier studies showed the presence of QTLs associated with milk fat percentage and yield on bovine chromosome 19 [72]. The QTL mapping identified FAS as a candidate gene responsible for some of the variation in milk fat percentage and yield [103]. The association study determined two SNPs located in exons 1 (g. 763 G>C) and 34 (g. 16009 A>G) that were associated with milk fat yield in dairy cattle

[102]. The SNP g. 16009 A>G caused a nonconservative substitution of threonine by alanine. Further studies showed association of SNPs in FAS gene with differences in milk fatty acid composition. For example, SNPs located in the FAS gene at positions 15531 (C>A) and 15603 (G>A) (accession AF285607) were associated with increased percentage of myristic acid (14:0) in milk for alleles A, respectively [101]. Another study looked at earlier discovered SNPs g. 16009 A>G [102] and g. 17924 A>G [101] in FAS gene that caused nonconservative substitutions of threonine by alanine in both cases and were associated with the concentration of myristic (14:0) acid in milk [92]. In addition, g. 16009 A>G and g. 17924 A>G were associated with the concentrations of linoleic ($18:2^{c9, c12}$) and oleic ($18:1^{c9}$) acids, respectively.

Milk lipid biosynthesis in the lactating mammary gland

The uptake of long-chain fatty acids from plasma into mammary epithelial cells is likely mediated by fatty acid transport proteins (FATPs, gene name: solute carrier family 27, SLC27) and fatty acid translocase (FAT/ CD36) [104-108]. There are six members of SLC27 family of FATPs. The FATP1, also known as SLC27A1, is highly expressed in adipose tissue and skeletal and heart muscles, and it translocates from inside a cell to the plasma membrane in response to insulin stimulation [104]. The FATP2 is expressed predominantly in liver and kidney, and FATP5 is mainly abundant in liver and plays a major role in fatty acid uptake by that organ. The only FATP protein found in small intestine is FATP4. There is not much information about the role of different FATPs in fatty acid uptake in mammary tissue. Regarding FAT/ CD36, it was shown that this fatty acid translocase is a key protein for long-chain fatty acid uptake in metabolically active tissues [105]. Thus, the absence of

murine FAT/ CD36 decreased uptake of fatty acid analogs in vivo in heart (50-80%), skeletal muscle (40-75%), and adipose tissue (60-70%).

After fatty acids enter a cell, they are either esterified to coenzyme A with a subsequent binding to acyl-CoA binding proteins (ACBP) or bound to fatty acid binding proteins (FABP) as NEFA. The FABPs are 14-15 kDa proteins that reversibly bind non-esterified saturated and unsaturated long-chain fatty acids, eicosanoids, and other lipids with the purpose of transporting or storing them inside a cell [109]. There are nine members of the FABP protein family that are expressed differentially in multiple tissues. The most abundant isoforms of FABPs expressed in the lactating bovine mammary gland are FABP3, FABP4, and FABP5 [107]. The FABP3 protein, also known as heart FABP, is expressed in tissues such as heart, skeletal muscle, mammary gland, brown adipose tissue, and others [109]. The main function of FABP3 in heart and skeletal muscles is to channel fatty acids inside a cell towards mitochondrial β-oxidation. The discovery of FABP3 protein in mammary gland was related to the identification of a mammary-derived growth inhibitor (MDGI) that turned out to be a mixture of FABP3 and FABP4 proteins [110, 111]. The MDGI inhibited the growth of human breast cancer cells [112]. The FABP3 protein is highly expressed in mammary gland during cell differentiation and formation of ductal structures at the onset of lactation [113], but the requirement of FABP3 for mammary tissue development and function was not well established [5, 114].

The FABP4, also known as adipocyte FABP, is highly expressed in mature and differentiating adipose tissues, and its expression is tightly regulated by fatty acids, peroxisome proliferator-activated receptor-γ agonists, and insulin [109]. There is not much information about FABP4 function in the mammary gland. The FABP5, also known as

epidermal FABP, is highly abundant in epidermis and also is expressed in adipose tissue, mammary gland, liver, and other tissues. The function of FABP5 in the mammary gland is not well known, but it was shown that the overexpression of FABP5 in benign rat mammary epithelial cell lines may induce metastasis [115].

Before fatty acids can be used in any of the metabolic pathways inside a cell, they have to be activated by esterification to the coenzyme A moiety [116]. Enzymes that catalyze this reaction are called acyl-CoA synthetases (ACS). There are different sub-families of ACS that are defined on the bases of the chain length of the acyl groups used as a substrate by those enzymes. In this review, I will only focus on long-chain acyl-CoA synthetases (ACSL), a sub-family that includes five members annotated as ACSL1, 3, 4, 5, and 6 with each member having multiple isoforms produced by alternative splicing [116]. There is not much information known about specific function and subcellular localization of each ACSL protein. The ACSL1, ACSL4, and ACSL5 are present in liver and adipose tissue, ACSL3 and ACSL6 are expressed in brain, ACSL4 is abundant in steroidogenic tissues, and ACSL5 is abundant in intestine [117]. The ACSLs are localized in a number of intracellular membranes including the plasma, microsomal, and mitochondrial outer membranes. One of the most studied ACSL is ACSL1, which, when overexpressed in the liver of rodents, increase TAG biosynthesis [117]. The other interesting function of ACSL1 is that it can interact with FATP1 in adipose tissue to channel fatty acids towards TAG biosynthesis, demonstrating a first example of vectorial acylation [118].

The glycerol phosphate pathway is the major pathway utilized by most cells for TAG biosynthesis [119]. The pathway consists of stepwise addition of activated fatty acyl groups to glycerol 3-phosphate (figure 2.1). The first and rate-limiting step in the TAG biosynthetic

pathway is catalyzed by a class of enzymes called glycerol-3-phosphate acyltransferases (GPAT) that add fatty acyl groups to the sn-1 position of glycerol 3-phosphate. In mammals, there are four known GPAT isoforms (GPAT1-4) that are localized to the mitochondrion (GPAT1-2) and endoplasmic reticulum (GPAT3-4) [120]. The mitochondrial GPAT isoforms account for about 50% of the total GPAT activity in rat liver whereas the microsomal GPAT isoforms account for 80-90% of the total GPAT activity in brown and white adipose tissues.

The GPAT1 is a 94 kDa protein that is embedded into the outer mitochondrial membrane and is resistant to N-ethylmaleimide (NEM). The GPAT1 catalyzes the esterification of SFA with a high preference for palmitic acid (16:0). The physiological significance of GPAT1 presence in the outer mitochondrial membrane is thought to be related to its involvement in channeling fatty acids away from β-oxidation and into glycerophospholipid biosynthesis by competing for fatty acids with carnitine palmitoyltransferase I [119]. The GPAT2 is another mitochondrial acyltransferase that is sensitive to NEM and is mostly abundant in testis but is also detectable in other tissues.

The GPAT4 is a 52 kDa NEM-sensitive protein that resides in the ER and catalyzes the acylation of lauric (12:0), palmitic (16:0), stearic (18:0), oleic ($18:1^{c9}$), linoleic ($18:2^{c9, c12}$), and arachidonic ($20:4^{c5, c8, c11, c14}$) acids to the sn-1 position of glycerol 3-phosphate [119]. The expression of GPAT4 is detected in most of the tissues but is highly abundant in brown and white adipose tissues, liver, testis, and different parts of the brain [120]. The GPAT4 was classified initially as 1-acylglycerol-3-phosphate acyltransferase-6 (AGPAT6) on the basis of its high amino acid similarity to AGPAT1 and AGPAT2 [121, 122]; later it was recognized as another microsomal GPAT and renamed as GPAT4 [123, 124]. The AGPAT6 knock-out mice had no subcutaneous adipose tissue and altered fatty acid composition of

glycerophospholipids, with an increased percentage of PUFA at the expense of MUFA [122]. During lactation, AGPAT6$^{-/-}$ mice had undeveloped mammary glands with a decreased number of lipid droplets [121]. The concentration of diacyglycerols (DAG) and TAG in milk of AGPAT6$^{-/-}$ mice was decreased by more than 90% compared with the milk from normal mice. Surprisingly, overexpression of GPAT4 did not increase TAG formation but rather increased biosynthesis of certain phospholipids [123, 124]. This observation might suggest the importance of GPAT4 in synthesizing phospholipids for the lipid droplet monolayer.

The second step in the glycerophospholipid biosynthetic pathway is catalyzed by AGPAT enzymes that add activated fatty acyl groups to the sn-2 position on the glycerol backbone, leading to the conversion of lysophosphatidic acid into phosphatidic acid (figure 2.1). There are 10 known isoforms of AGPAT enzymes (AGPAT1-10), but only AGPAT1 and AGPAT2 are well studied and considered to contribute significantly to the functionality of TAG biosynthetic pathway. The AGPAT6 and AGPAT10 were discovered to have GPAT activity and subsequently renamed GPAT4 and GPAT3, respectively [119]. The AGPAT1 is a 32 kDa microsomal protein that has a preference for lauric (12:0), myristic (14:0), palmitic (16:0), palmitoleic (16:1^{c9}), linoleic (18:2$^{c9, c12}$), and α-linolenic (18:3$^{c9, c12, c15}$) acids, followed by stearic (18:0), oleic (18:1^{c9}), and arachidonic (20:4$^{c5, c8, c11, c14}$) acids and poor affinity for arachidic (20:0) and lignoceric (24:0) acids as substrates [125, 126].

Mouse AGPAT1 is expressed in most tissues including muscle, white and brown adipose tissues, liver, and heart [119]. Overexpression of AGPAT1 in 3T3-L1 adipocytes promotes non-insulin-stimulated exogenous oleate (18:1^{c9}) uptake and incorporation into phosphatidic acid and glucose uptake and conversion into fatty acids [127]. The AGPAT1 overexpression suppresses NEFA release during stimulated lipolysis without affecting

glycerol release, suggesting a role for AGPAAT1 in stimulating NEFA reesterification. In C_2C_{12} myotubes, AGPAT1 overexpression increases insulin-stimulated oleate ($18:1^{c9}$) uptake and glucose partitioning towards fatty acids [127].

The AGPAT2 enzyme is also a microsomal 31 kDa protein that catalyzes the acylation of myristic (14:0), palmitic (16:0), oleic ($18:1^{c9}$), and linoleic ($18:2^{c9, c12}$) acids and, to a lesser extent, the acylation of stearic (18:0) and arachidonic ($20:4^{c5, c8, c11, c14}$) acids to 1-oleoyl-lysophosphatitidc acid [126]. The fatty acid preference of AGPAT2 suggests that it might be involved mainly with de novo glycerophospholipid biosynthesis because only those compounds have the sn-2 position occupied by oleic ($18:1^{c9}$) or linoleic ($18:2^{c9, c12}$) acid and not by arachidonic acid ($20:4^{c5, c8, c11, c14}$) as is the case with membrane phospholipids. The AGPAT2 is expressed at high levels in visceral white and brown adipose tissues, liver, heart, and pancreas, at moderate levels in muscle, lung, and small intestine, and at low levels in brain, placenta, and subcutaneous adipose tissue [119]. The deficiency of AGPAT2 is linked to congenital generalized lipodystrophy that occurs in humans and was studied in mouse using transgenic models [128, 129]. The disease is characterized by lack of adipose tissue at birth, severe insulin resistance, hypertriglyceridemia, hepatic steatosis, and earlier onset of diabetes.

The phosphatidate produced through the action of AGPAT enzymes has to be dephosphorylated to produce DAG before advancing to the final steps of TAG and phospholipid biosynthesis. The enzyme catalyzing the removal of a phosphate group from phosphatidic acid is called phosphatidate phosphatase (PAP) or lipin [130], (figure 2.1). There are four known lipin isoforms (lipin1A, lipin1B, and lipin2-3) encoded by three different genes. Lipin-1A and -1B are produced from the same gene by differential splicing.

In addition to being a PAP, lipin-1 also acts as a transcriptional coactivator. The lipins are localized in the cytosol and translocate to the ER in response to elevated intracellular concentrations of fatty acids [131]. The main sites of lipin-1 expression are adipose tissue, muscles, and liver, with lipin-2 being expressed at high levels in liver, brain, and kidney and lipin-3 being detected in small intestine and liver [132].

The gene encoding lipin-1 was discovered by studying a spontaneous mouse mutation known as fatty liver dystrophy (fld) [133]. During the neonatal period, the fld mouse was characterized by a complex phenotype that included fatty liver, hypertriglyceridemia, and lipodystrophy affecting white adipose tissue and interscapular brown adipose tissue [133, 134]. On the other hand, a transgenic mice with enhanced expression of lipin-1 in adipose tissue developed obesity [135].

The final step in TAG biosynthesis is catalyzed by diacylglycerol acyltransferases (DGAT) that add activated fatty acyl groups to the sn-3 position on the glycerol backbone (figure 2.1). There are two known isoforms of the enzyme: DGAT1 and DGAT2 [136]. Both DGAT isoforms reside in the ER, and a deficiency in either of the isoforms was associated with decreased TAG concentration in affected tissues. The substrate specificity of DGAT isoforms was tested in competition assays that revealed DGAT1 preference for oleic acid ($18:1^{c9}$) over palmitic acid (16:0), with DGAT2 not showing any specific preferences at all [95]. In another study, DGAT2 purified from a fungus showed substrate specificity towards medium-chain fatty acids as compared with long-chain fatty acids and catalyzed the addition of activated fatty acyl groups to DAG containing short- and medium-chain fatty acyl groups more readily than to DAG containing longer fatty acyl groups [137].

Over the past few decades, a group of sterol regulatory element binding proteins (SREBPs), consisting of three transcription factors (SREBP-1a, SREBP-1c, and SREBP-2) that bind to sterol response element (SRE) on the promoters of different genes, was studied extensively because of the ability of SREBPs to transcriptionally regulate genes from fatty acid and cholesterol biosynthetic pathways [138]. The process of converting SREBP proteins from an inactive to a transcriptionally active form called the SREBP pathway will be the next focus of this review (figure 2.2).

The SREBP proteins (SREBP-1a, SREBP-1c, and SREBP-2) are encoded by two different genes (SREBP1 and SREBP2) with SREBP-1a and SREBP-1c being produced from SREBP1 gene by alternate transcription [41]. The SREBP proteins are embedded into the ER membrane, with their N-terminal transcriptionally active and C-terminal regulatory domains projecting into the cytosol. Another protein named as SREBP cleavage activating protein (SCAP) also resides in the ER membrane and interacts through its C-terminal domain containing multiple WD40 sequences with the C-terminal regulatory domain of SREBP proteins on the cytosolic site of the ER [41].

In sterol-depleted cells, SCAP transports SREBP proteins in coated vesicles from ER to Golgi, where the N-terminal domain of SREBP proteins is released into cytosol after a sequence of proteolytic cleavages catalyzed by S1P and S2P proteases (figure 2.2). The cleaved SREBP proteins then enter the nucleus where they activate transcription of genes for fatty acid and cholesterol biosynthetic pathways. When ER cholesterol concentration rises above 5%, SCAP binds cholesterol and undergoes a conformational change that prevents SCAP-SREBP transport via coated vesicles to Golgi. As a result, N-terminal domain of

SREBP proteins cannot be released and no transcriptional activation of SRE-containing genes can occur.

Yet another protein, named as insulin-induced gene (Insig) was found to reside in the ER membrane and to bind to SCAP in the presence of oxysterols, leading to the inhibition of SCAP-SREBP transport to Golgi, even in sterol-depleted cells (figure 2.2). There are two known genes (Insig-1 and Insig-2) that code for three different forms of Insig proteins (Insig-1, Insig-2a, and Insig-2b). In the presence of oxysterols, Insig-1 not only prevents SCAP-SREBP transport to Golgi but also binds hydroxymethylglutaryl-CoA on the cytosolic side of ER to facilitate its proteolytic degradation. The Insig-1 gene also is activated transcriptionally with SREBPs, thus providing a feed-back mechanism by which nuclear SREBP trigger buildup of their own inhibitor.

Whereas SREBP-2 protein participates in the regulation of cholesterol biosynthesis and SREBP-1a participates in the regulation of both cholesterol and fatty acid biosynthesis, the SREBP-1c is involved mainly with the regulation of lipogenic genes. The SREBP-1c is expressed in most of the tissues in mice and humans, with high levels of expression in the liver, white and brown adipose tissues, adrenal gland, and brain [139]. In contrast to SREBP-1a and SREBP-2, expression and nuclear abundance of SREBP-1c does not depend on cellular cholesterol concentration [140]. Moreover, overexpression of the nuclear form of SREBP-1c in the liver of transgenic mice caused TAG accumulation without any change in cholesterol concentration [141].

The activity of SREBP-1c is controlled by transcriptional regulation of its gene expression and by proteolytic cleavage of SREBP-1c in Golgi. It was shown that in the liver insulin can increase SREBP-1c proteolytic activation by decreasing the mRNA abundance of

Insig-2a that otherwise would be able to block SCAP-SREBP-1c transport from ER to Golgi and subsequently inhibit SREBP-1c activation [142]. Insulin did not change the transcription of Insig-1, suggesting that Insig-1 is not involved in the regulation of fatty acid biosynthesis through SREBPs.

Transcriptional regulation of SREBP-1c is accomplished by liver X-activated receptors (LXR), insulin, and glucagon [143]. The main role of LXRs is to activate oleate ($18:1^{c9}$) production for the synthesis of cholesteryl esters. This activation could be potentially achieved by up-regulating fatty acid elongase and the stearoyl-CoA desaturase activities as it was observed in transgenic mice overexpressing the nuclear form of SREBP-1a in their livers [144, 145]. LXRs are also a part of the regulatory mechanism by which UFA can suppress fatty acid biosynthesis [143]. Insulin also up-regulates SREBP-1c expression, leading to an increase in the rates of fatty acid biosynthesis as demonstrated in rodents using cultures of isolated hepatocytes and adipocytes [146]. The effects of insulin on SREBP-1c expression are thought to be mediated through PI3-kinase pathway, with potential downstream effectors being protein kinase B and protein kinase C-λ. Glucagon is known to oppose the effects of insulin on SREBP-1c expression via cAMP.

The information regarding the regulation of fatty acid and cholesterol biosynthesis by SREBPs in the lactating mammary gland is limited. In mice, it was shown that SREBP-1a and SREBP-1c are expressed in mammary tissue and their expression is increased during lactation [147]. In addition to earlier described regulatory mechanisms for lipid biosynthesis involving SREBP-1c that are assumed to be true in the lactating mammary gland, some studies suggested a potential role for insulin in the regulation of lipid biosynthesis via PI3 kinase pathway, with protein kinase B (Akt1) being a possible downstream effector (figure

2.2). Overexpression of a constitutively active form of Akt1 in mice during lactation results in precocious lipid droplet formation in mammary epithelial cells, with a significant increase in milk lipid concentration that reaches 60-70% on a wet weight basis as compared with only 25-30% in wild type mice [148]. On the basis of recent discoveries of the Akt role in the regulation of SREBP activation [149-151], it was proposed [147] that the insulin-mediated up-regulation of lipid biosynthesis via Akt is achieved by either Akt-stimulated proteolytic activation of SREBP-1c or an Akt-stimulated decrease in the degradation of a nuclear form of SREBP-1c in the nucleus or both (figure 2.2).

A study on a diet-induced MFD in dairy cows highlighted a potential role of SREBP-1 in the regulation of lipid biosynthesis in lactating mammary gland by showing a reduction in SREBP-1, SCAP, Insig-1, and Insig-2 gene expressions after feeding a diet rich in vegetable oil [55]. On the basis of the results of this study, it is difficult to determine, however, whether Insig-1 or Insig-2 played any role in the regulation of SREBP-1 activation, because, if it were the case, the expression of either Insig-1 or Insig-2 or both isoforms should have been increased. It also could be possible that the low expression levels for Insig-1 and Insig-2 were as a result of decreased SCAP expression. In another study, there was an up-regulation in the expression of SREBP-1, SREBP-2, SCAP, Insig-1, and Insig-2 during lactation, but the information is still too incomplete to make any conclusions about potential involvement of any of the above proteins from the SREBP pathway in the regulation of lipid biosynthesis [108].

One of the first studies looking at the global changes in lipid metabolism in the mammary gland during lactation was performed in mice by using a combination of microarray analysis and metabolic profiling [106]. Later, a similar study was conducted in

dairy cows [108]. The first set of genes that was evaluated in both studies was related to the regulation of fatty acid uptake by the mammary gland. Fatty acids in blood are present as NEFA bound to albumin and as a part of very-low density lipoproteins (VLDL) where they are esterified into TAG. Lipoprotein lipase (LPL) is an enzyme that is highly expressed in mammary gland [152, 153] and responsible for the hydrolysis of TAG from VLDL with the release of NEFA and glycerol. The LPL gene expression was up-regulated in both murine and bovine mammary glands during lactation, and, for the bovine mammary gland, the change in activity for LPL over time resembled a lactation curve, pointing to the importance of LPL and VLDL in supplying fatty acids to the mammary gland during lactation in dairy cows [106, 108]. Recent evidence indicates the importance of a VLDL receptor in facilitating the interaction between LPL and VLDL [154] and the requirement of this interaction for efficient TAG hydrolysis. The expression of the VLDL receptor in mammary gland was up-regulated during lactation in dairy cows [108].

In mice, three genes from the SLC27 family of fatty acid transporters (SLC27A1, SLC27A3, and SLC27A4) were expressed in mammary tissue and their expression was up-regulated with the onset of lactation [106]. The SLC27A6 transporter was the only one from the SLC27 family to be significantly up-regulated in bovine mammary gland during lactation [107, 108]. The fatty acid translocase (CD36) gene thought to be involved in fatty acid transport [155] was up-regulated during lactation in both mouse and cow mammary glands. Even though the definite role for CD36 involvement in fatty acid transport has not been established, the fact that CD36 co-localizes with SLC27A6, ACSL, and FABP proteins [156] makes CD36 a good candidate for the regulation of lipid biosynthesis in the mammary gland.

The next set of genes involved in lipid biosynthesis in a lactating mammary gland is associated with fatty acid activation and channeling inside a lipogenic cell. The ACSL1 that catalyzes the activation of long-chain fatty acids is highly expressed in bovine mammary tissue [107], and its expression increases more than four-fold at the onset of lactation [108]. Among a group of ACSS enzymes that catalyze the activation of medium-chain fatty acids, ACSS1 and ACSS2 are the most abundant in bovine mammary tissue and their expression is increased during lactation [108]. The activated fatty acids in the form of acyl-CoA are transported by acyl binding proteins (ACBP) to their final destination within a cell, but in the mammary gland the role of ACBP proteins in lipid metabolism is considered to be minor and some of the ACBP protein functions are considered to be fulfilled by FABPs [106, 108].

Non-activated fatty acids are bound and transported inside a cell by FABPs. The FABP3 is the most abundant isoform in both murine and bovine mammary tissues, but other FABP isoforms such as FABP4 and FABP5 are present as well [106, 108]. The FABP3 was the second most abundant transcript in bovine mammary gland, and its expression was up-regulated tremendously during lactation [107, 108]. The expression of FABP4 and FABP5 also was up-regulated with the onset of lactation but to a lesser extend compared with FABP3. It was speculated that FABP3 provides substrates for SCD, and FABP4 binds oleic ($18:1^{c9}$) acid, the major product of the SCD-catalyzed reaction and then transports the oleate ($18:1^{c9}$) to other enzymes for TAG biosynthesis [108]. In the bovine mammary gland, FABP3 also buffers the cells from negative effects of activated fatty acyl groups that might otherwise inhibit the activity of SCD and ACACA enzymes, playing the role that was traditionally attributed to acyl fatty acid binding proteins [157].

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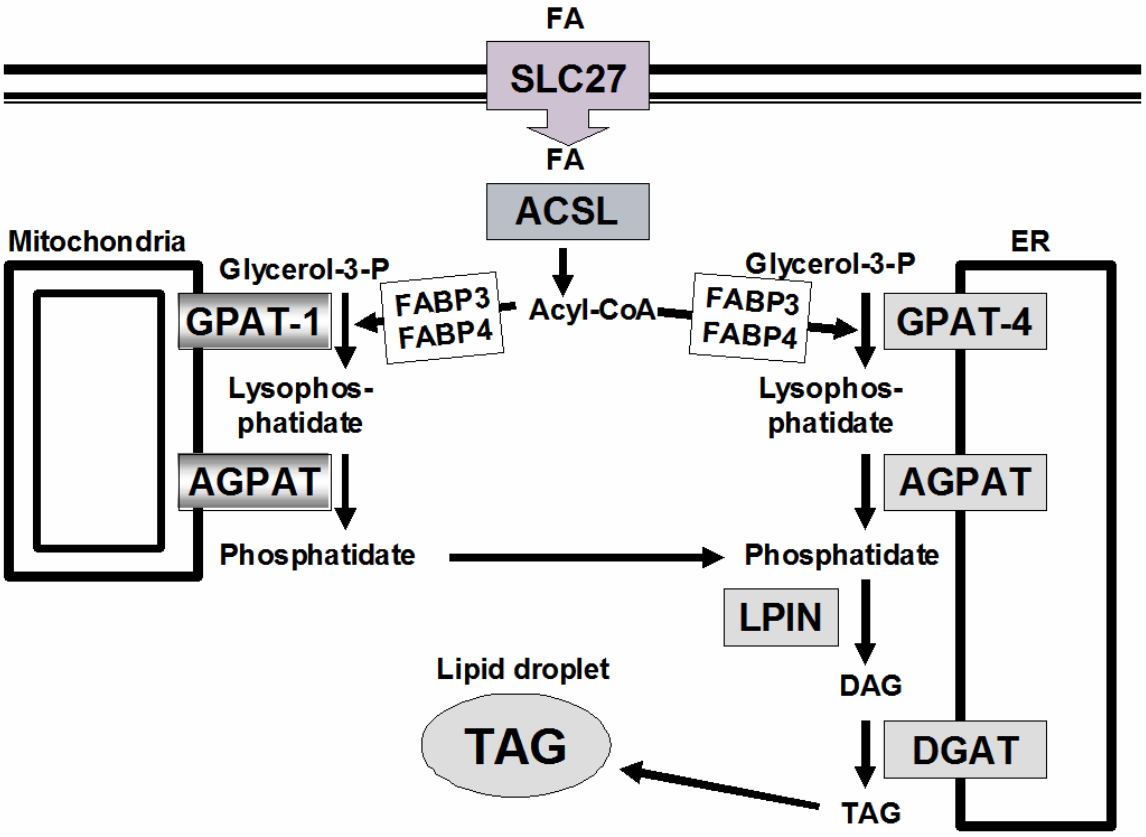

Figure 2.1 TAG biosynthetic pathway. ACSL, long-chain acyl-CoA synthetase; AGPAT, 1-acylglycerol-3-phosphate acyltransferase; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; FA, fatty acid; FABP3 and FABP4, fatty acid binding proteins 3 and 4; GPAT-1 and GPAT-4, glycerol-3-phosphate acyltransferases 1 and 4; LPIN, phosphatidate phosphatase; SLC27, solute carrier protein family 27; TAG, triacylglycerol.

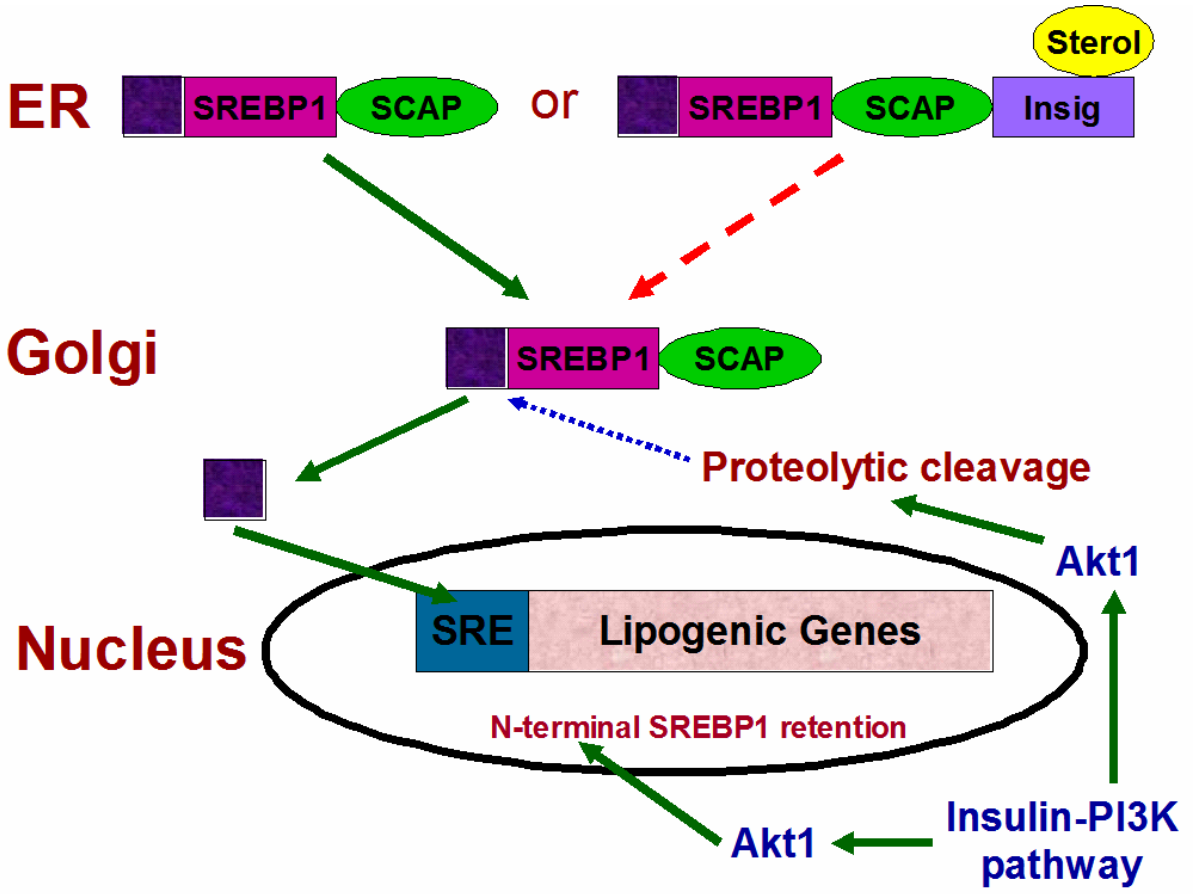

Figure 2.2 Regulation of lipogenesis in mammary epithelial cells by the SREBP pathway and insulin. Akt1, protein kinase B; ER, endoplasmic reticulum; Insig, insulin-induced gene; PI3K, phosphoinositide-3-kinase; SRE, sterol response element; SREBP1, sterol regulatory element binding protein 1; SCAP, SREBP cleavage-activating protein.

CHAPTER 2. EFFECTS OF POLYMORPHISMS IN GPAT1, GPAT4, AGPAT1, AND LPIN1 GENES FROM THE TRIACYLGLYCEROLE BIOSYNTHETIC PATHWAY ON MILK FATTY ACID COMPOSITION IN DAIRY CATTLE

A paper to be submitted to BMC Genomics

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Abstract

Developing tools to improve the healthfulness of milk through genetic selection is the goal of our study. High concentrations of saturated fatty acids (SFA) in milk including the atherogenic fatty acids such as palmitic (16:0) and myristic (14:0) are detrimental to human health and as such are not desirable. Increasing the concentration of unsaturated fatty acids (UFA) in milk at the expense of SFA, however, is beneficial to human health. The objectives of our study were to discover genetic polymorphisms in glycerol-3-phosphate acyltransferases-1 and -4 (GPAT1 and GPAT4), 1-acylglycerol-3-phosphate acyltransferase-1 (AGPAT1) and a phosphatidate phosphatase (LPIN1) and to test the associations of those polymorphisms with bovine milk fat percentage and fatty acid composition. We hypothesized that because of the selectivity in fatty acid acylation by different acyltransferases and the importance of LPIN1 for the triacylglycerol

biosynthesis in adipose tissue, polymorphisms in GPAT1, GPAT4, AGPAT1, and LPIN1 genes will be associated with differences in bovine milk fat percentage and fatty acid composition. The results of our study showed that the overall haplotype effect of GPAT4 was associated significantly with the concentrations of capric (10:0), lauric (12:0), palmitic (16:0), and oleic ($18:1^{c9}$) acids and as a consequence with the concentrations of SFA, UFA, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), SFA/UFA, C16 and C18 desaturation indices, and an atherogenic index (AI). The overall haplotype effect of GPAT1 was significantly associated with milk fat percentage, the concentrations of caproic (6:0), caprylic (8:0), capric (10:0), tridecylic (13:0), margaric (17:0), myristoleic ($14:1^{c9}$) acids, and C14 desaturation index. The overall haplotype effect of AGPAT1 showed significant associations with the concentrations of PUFA, caproic (6:0), margaric (17:0), linoleic ($18:2^{c9, c12}$) acids, conjugated linoleic acid (CLA, $18:2^{c9, t11}$), and myristoleic ($14:1^{c9}$) acid. The overall haplotype effect of LPIN1 was associated significantly only with myristoleic ($14:1^{c9}$) acid concentration and C14 desaturation index. In conclusion, the identified polymorphisms in GPAT1, GPAT4, AGPAT1, and LPIN1 can be used as genetic markers by animal breeders to select for animals producing milk with healthier fatty acid composition and with higher fat percentage.

Introduction

The regulation of bovine milk fatty acid composition by dietary and genetic means received much attention in recent years because of the adverse effects of certain dietary fatty acids on human health [1-6]. The total cholesterol (TC), low-density lipoprotein

cholesterol (LDL-C), and high-density lipoprotein cholesterol concentrations in plasma are predictors of cardiovascular disease risk [7], and those concentrations are influenced by a number of factors including dietary fatty acids. Saturated fatty acids (SFA) as a class and individual SFA such as palmitic (16:0), myristic (14:0), and lauric (12:0) acids raise plasma TC and LDL-C concentrations much higher than other fatty acids, and their presence at high concentrations in human diets is undesirable [8]. Monounsaturated fatty acids (MUFA), however, are considered to be neutral with respect to plasma cholesterol concentrations, and their concentrations in milk can be increased at the expense of SFA to make milk healthier. Improving fatty acid composition of bovine milk by dietary means has limitations because of ruminal biohydrogenation of unsaturated fatty acids (UFA) [9], but the genetic regulation of bovine milk fatty acid composition is plausible because of the moderate to high heritabilities of milk fatty acids [3, 10-13]. Thus, the objective of our study was to develop genetic markers that will allow selection for animals producing milk with healthier fatty acid profile.

The biosynthesis of milk triacylglycerols (TAG) occurs in mammary epithelial cells through a stepwise addition of activated fatty acyl groups to glycerol 3-phosphate by different acyltransferases [14]. The first and rate limiting step in the TAG biosynthetic pathway is catalyzed by glycerol-3-phosphate acyltransferases (GPAT) that add fatty acyl groups to the sn-1 position of glycerol 3-phosphate. The major GPAT isoforms known to be expressed in bovine mammary gland are GPAT1 (mitochondrial) and GPAT4 (microsomal) [15]. The GPAT1 catalyzes the acylation of saturated fatty acids with a high preference for palmitic acid (16:0) whereas GPAT4 catalyzes the acylation of lauric (12:0), palmitic (16:0), stearic (18:0), oleic ($18:1^{c9}$), linoleic ($18:2^{c9, c12}$), and arachidonic

($20:4^{c5, c8, c11, c14}$) acids [14]. The GPAT4 was initially classified as 1-acylglycerol-3-phosphate acyltransferase-6 (AGPAT6) based on high amino acid similarity to AGPAT1 and AGPAT2 [16, 17], but later was recognized as another microsomal GPAT and renamed as GPAT4 [18, 19] . The AGPAT6 knock-out mice had no subcutaneous adipose tissue and altered fatty acid composition of glycerophospholipids with increased percentage of polyunsaturated fatty acids (PUFA) at the expense of MUFA [17]. During lactation, $AGPAT6^{-/-}$ mice had underdeveloped mammary glands with reduced number of lipid droplets [16]. The concentration of diacyglycerols (DAG) and TAG in milk of $AGPAT6^{-/-}$ mice was decreased by more than 90% compared with the milk from normal mice. Surprisingly, overexpression of GPAT4 did not increase TAG formation but rather increased biosynthesis of certain phospholipids [18, 19].

The second step in the TAG biosynthetic pathway is catalyzed by AGPAT that add fatty acyl groups to the sn-2 position on the glycerol backbone, leading to the conversion of lysophosphatidic acid into phosphatidic acid. The AGPAT1 has a preference for lauric (12:0), myristic (14:0), palmitic (16:0), palmitoleic ($16:1^{c9}$), linoleic ($18:2^{c9, c12}$), and α-linolenic ($18:3^{c9, c12, c15}$) acids followed by stearic (18:0), oleic ($18:1^{c9}$), and arachidonic ($20:4^{c5, c8, c11, c14}$) acids and poor affinity for arachidic (20:0) and lignoceric (24:0) acids as substrates [20, 21]. The phosphatidate produced through the action of AGPAT has to be dephosphorylated to produce DAG before advancing to the final steps of the TAG biosynthesis. The enzyme catalyzing the removal of a phosphate group from phosphatidic acid is called phosphatidate phosphatase (PAP) or lipin (LPIN) [22]. The LPIN are localized in the cytosol and translocate to ER in response to elevated intracellular concentrations of fatty acids [23]. The major isoform of PAP expressed in

bovine mammary gland is LPIN1 [15]. In addition to being a PAP, LPIN1 also acts as a transcriptional coactivator.

The positional distribution of fatty acids in milk TAG is not random, and it depends on the specificity of different acyltransferases for a particular fatty acid. The sn-1 position of bovine milk TAG, for example, is occupied predominantly by palmitic (16:0; 34.0 mol %) and oleic ($18:1^{c9}$; 30.0 mol %) acids followed by stearic (18:0; 10.5 mol %), myristic (14:0; 9.7 mol %), and lauric (12:0; 4.9 mol %) acids [24]. The major fatty acids at the sn-2 position are palmitic (16:0; 32.3 mol %) and oleic ($18:1^{c9}$; 18.9 mol %) acids followed by myristic (14:0; 17.5 mol %), stearic (18:0; 9.5 mol %), and lauric (12:0; 6.2 mol %) acids. Butyric (4:0; 35.4 mol %), oleic ($18:1^{c9}$; 23.1 mol %), and caproic (12.9 mol %) acids are the major fatty acids at the sn-3 position.

Considering the positional distribution of fatty acids in milk TAG and preferential acylation of different fatty acids by acyltransferases, we hypothesize that genetic polymorphisms in GPAT1, GPAT4, AGPAT1, and LPIN1 genes will be associated with differences in milk fatty acid composition. To test the hypothesis, single nucleotide polymorphisms (SNPs) in GPAT1, GPAT4, AGPAT1, and LPIN1 genes were identified by sequencing. After genotyping animals for the discovered SNPs, intragenic haplotypes were reconstructed and the associations between the haplotypes within the same gene and milk fatty acid composition were tested. The results of the association tests are reported in this paper.

Materials and methods

Milk fatty acid analysis

Milk samples were collected during morning milking once a month throughout a 305-d lactation period and stored at -20 °C until further analysis. The extraction of milk fatty acids with subsequent esterification into methyl esters for the analysis by gas chromatography (GC) was performed according to the procedure published by Chouinard et al. [25] with minor modifications. The procedure is based on the original milk lipid extraction method with hexane and isopropanol developed by Hara and Radin [26] and fatty acid methyl ester production method developed by Christie [24]. After thawing, milk samples were vortexed for 10 s and 25 mL aliquots were transferred to 50 mL teflon centrifuge tubes pre-rinsed with chloroform/methanol (2/1 vol.vol). Following centrifugation at 17,800 × *g* for 30 min at 4 °C, the liquid phase of the milk samples was removed and 18 mL of hexane/isopropanol (3/2 vol/vol) were added for every gram of lipids with subsequent vortexing for 1 min. To achieve a better separation of hexane phase containing lipids from water phase containing proteins, 12 mL of 6.7 % sodium sulfate (Na_2SO_4) solution were added for every gram of lipids and samples were vortexed for 1 min. After centrifugation at 2,500 × *g* for 5 min at 4 °C, the hexane fraction containing lipids was removed into a scintillation vial pre-rinsed with chloroform/methanol (2/1 vol/vol) and an aliquot containing 40 mg of lipids (400 μL) was transferred into an esterification vial . After the evaporation of hexane under nitrogen gas, 2 mL of hexane were added to the esterification vial followed by 40 μL of methyl acetate and vortexing for 30 s. The methylation step was initiated by the addition of 40 μL of sodium methoxide (0.4 mL of 5.4 M sodium methoxide in 1.75 mL of methanol)

solution. The mixture was vortexed for 30 s and allowed to react for 10 min at room temperature. The reaction was terminated by the addition of 60 μL of oxalic acid (1 gram of oxalic acid/30 mL of diethyl ether) solution. The samples were centrifuged at 2,400 × *g* for 5 min at 4 °C, and a clear hexane fraction, which contained fatty acid methyl esters, was placed into GC vials, purged with nitrogen gas for 3 s, capped, and used for chromatographic analysis. In the above steps, capping of the vials that contained hexane lipid extract was preceded by purging with nitrogen gas for 3 s.

A Varian 3900 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA) equipped with CP-8400 auto-sampler, CP-8410 auto-injector, CP-1177 split/splitless injector, and flame ionization (FID) detector was used to analyze fatty acid methyl esters with helium as a carrier gas. One μL of fatty acid methyl esters in hexane was injected (split ratio 50:1) into a fused silica capillary column (Supelco^{TM}-2560 Capillary Column, 100m x 0.25 mm i.d., with 0.2 μm film thickness) with the initial column temperature set at 70 °C and held for 4 min. Then, the column temperature was increased to 175 °C at the rate of 13 °C/min and held for 27 min with subsequent increase to 215 °C at the rate of 4 °C/min where it was held for 28 min [27]. The injector and detector temperatures were set constant at 220 °C. Peak area was measured by integration using Star Chromatography Workstation Version 6, and peaks were identified by comparing the retention times with separately run fatty acid methyl ester standards (Matreya LLC, Pleasant Gap, PA).

DNA extraction

Genomic DNA was extracted from 20 mL of blood obtained from the coccygeal vein of every dairy cow. The blood was collected into 10 mL EDTA Vacutainer tubes and kept at 4 °C. After transferring 20 mL of blood into 50 mL centrifuge tubes, the ice-cold 1X red blood cell (RBC) lysis buffer was added to fill in the tubes that were inverted and incubated on ice for 30 min. The samples were centrifuged at 300 × *g* for 5 min at 4 °C. Supernatant was aspirated and 20 mL of ice-cold 1X RBC lysis buffer were added again followed by a light vortexing and 30 min incubation on ice. After centrifugation and aspiration steps performed as mentioned earlier, 20 mL of room temperature PBS buffer were added to wash the pellet and the centrifugation step was repeated again. Then, supernatant was discarded and pellet was resuspended in PBS, transferred to cryovial, and stored at -70 °C until further analysis. The DNA purification from the white blood cells was performed with DNeasy® Blood and Tissue Kit (Qiagen Inc., Valencia, CA) according to the instructions. The DNA concentration was measured using NanoDrop® ND1000, and dilutions were made with DNase-free water to desired concentration.

SNP discovery and genotyping

The GPAT4, GPAT1, AGPAT1, and LPIN1 genes from the TAG biosynthetic pathway (table 2.1), were sequenced in exonic and some intronic regions to discover SNPs. A set of 12 DNA samples from cows that were daughters of 12 different sires was used for the SNP discovery. The PCR primers were designed with Primer3 (version 0.4.0) software [28]. Regular or “Hot-start” PCR was performed to amplify the DNA regions of interest. For the regular PCR, the reaction mixture contained 50 ng of genomic DNA, 13.6 μL of

DNA grade water, 2 μL of dimethyl sulfoxide (DMSO), 1.5 μL of $MgCl_2$ (25 mM), 0.1 μL of forward and reverse primers (100 ng/μL), 0.5 μL of dNTP mix (40 mM), 5 μL of 5X buffer, and 0.2 μL of Tag DNA polymerase (5 U/μL) with the total volume of the reaction mixture equal to 25 μL. For the "Hot-start" PCR, the reaction mixture was almost the same as for the regular PCR with the only difference that 5 μL of 10X buffer was used instead of 5X buffer and 0.2 μL of " Hot-start" Tag DNA polymerase (5 U/μL) was used instead of Tag DNA polymerase. The PCR were performed in a DNA Engine thermal cycler (Bio-Rad) with the separate temperature cycle programs for the regular and "Hot-start" PCR. The regular PCR was performed with the following program: 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, an optimal annealing temperature for a particular primer set (table 2.2) for 30 s, and 72 °C for 30 s with a final extension step at 72 °C for 10 min. The "Hot-start" PCR was performed with the following program: 95 °C for 15 min followed by 30 cycles of 94 °C for 1 min, an optimal annealing temperature for a particular primer set (table 2.2) for 1 min, and 72 °C for 1 min with a final extension step at 72 °C for 10 min. The optimal annealing temperature for a particular primer set was determined by a PCR temperature gradient. Before sequencing, the PCR product was cleaned up from unused primers and dNTPs with ExoSAP-IT® (USB Corporation, Cleveland, OH) that contained exonuclease 1 and shrimp alkaline phosphatase [29]. Reverse and forward sequences representing 12 different samples were aligned using Vector NTI Advance™ 10 to identify SNPs. Genotyping of discovered SNPs was performed with the Sequenom MassARRAY platform using 10 ng of genomic DNA dissolved in DNase-free water [30]. Haplotypes and their frequencies were estimated using PHASE (version 2.1) program [31, 32]. Only samples with the

probability ≥0.9 for the best haplotype pair were used in the analysis. The restriction fragment length polymorphism technique was used to genotype animals for DGAT1 A232K mutation. A set of primers (table 2.2), was used to amplify a 405-bp region of DNA containing the mutation site by using the "Hot start" PCR. The resulting PCR product then was digested with EaeI restriction enzyme and run on a 2% agarose gel. There were 230-bp and 175-bp DNA fragments on a gel when an animal had DGAT1 232K genotype and a 405-bp DNA fragment on a gel when an animal had DGAT1 232A genotype.

Statistical analysis

A linear mixed model for longitudinal data was used to analyze the data with PROC MIXED procedure of SAS 9.1 (2002). The haplotype substitution model [33] used to test the association between intragenic haplotypes and milk fatty acid composition was the following:

$Y = \mu + dim + dim^2 + dim^3 + cg + dim*cg + dim^2*cg + dim^3*cg + \sum b_k H_k + sire + \varepsilon$

where y is the response variable; μ is the mean response at 0 dim for a cow with no copies of the haplotypes; dim, dim^2, and dim^3 are covariates describing the effects of days in milk on milk fatty acid composition; cg is a fixed effect of the contemporary group (cg=8 classes); H_k is a haplotype effect fitted as a covariate and coded as 0, 1, or 2 for 0, 1, or 2 copies of each haplotype present in an animal; b_k is the partial regression coefficient which corresponds to the haplotype substitution effect for the k^{th} haplotype as a deviation from the effect of the most frequent haplotype that is set to zero to have a full rank model; sire is a sire random effect; and ε is a residual error. The higher order terms

for days in milk were introduced to account for a non-linear trend of the response variables over time. The cubic term of days in milk allows the response variable to have different slopes whenever the direction of its trend changes over time. When the higher order terms for days in milk were not significant, they were dropped from the model. Contemporary groups were created by combining herd (two herds) and season of calving (four seasons: December to February, March to May, June to August, and September to November) effects. The year of sampling was not included in the model to avoid redundancy because the season of calving is already containing some information about the year when a particular sample was collected. Haplotypes with a population frequency more than 0.05 were used in the analysis, and those with frequency less than 0.05 were pooled in the "other" category. The CONTRAST statement in PROC MIXED was used to construct the F-tests of the overall haplotype effects, and, when the test was significant after applying the Bonferroni adjustment to account for multiple testing, the pairwise comparisons between all haplotypes within each gene were performed using the ESTIMATE statement and the estimates of the differences between haplotype effects, their standard errors, and p-values were reported. The REPEATED statement was used to indicate that milk samples were collected repeatedly for the same animal throughout the 305-d lactation period. Correlations between repeated measures taken on the same animal were modeled using a first order autoregressive covariance structure that assumes equal variances and correlations that decline exponentially with an increase in the distance between time points [34]. The covariance structure was selected on the basis of the biology of the experiment and using Akaike's and Bayesian Information Criteria and residual log likelihood [35].

A Bonferroni adjustment was used to control type I error rate during multiple comparisons between intragenic haplotypes. Bonferroni-adjusted significance levels were determined by dividing desired experiment-wide significance level (0.05) by the number of pairwise comparisons. The numbers of pairwise comparisons for GPAT4, GPAT1, AGPAT1, and LPIN1 genes were 15, 15, 10, and 21, respectively that resulted in the Bonferroni-adjusted significance levels of 0.0033, 0.0033, 0.0050, and 0.0024, respectively. We compared haplotype sequences within each gene on the basis of their effects on milk fatty acid composition to determine "tag" SNPs that can be used as genetic markers.

In recent years, a number of studies evaluated the effects of SNPs from different genes on milk fatty acid composition [1, 6, 36, 37]. It is difficult, however, to compare the size of the effects of different polymorphisms on any particular fatty acid concentration in milk because of the different approaches used to analyze the data. To compare the results of our study with earlier published data, we genotyped our animals for diacylglycerol acyltransferase-1 (DGAT1) K232A and fatty acid synthase g. 17924A>G polymorphisms and analyzed the data the same way as earlier, with the only difference that allele effects of the SNPs were tested instead of the haplotype effects. All animals on the study were treated in accordance with guidelines established by the Iowa State University Committee on Animal Care.

Results

Mean values, their standard deviations, and 5% and 95% quantiles for milk production, milk fat percentage, concentrations of different fatty acids and fatty acid groups, and fatty

acid indices are reported in table 2.3. There were 39 different milk fatty acids measured by GC. All fatty acid data were used to calculate the concentrations of individual fatty acids that were expressed as wt %, but the data reported are only for fatty acids present in milk at relatively high concentrations. The mean values for the major milk fatty acids in our study were similar to those reported by others [2, 13]. The major fatty acid in milk was palmitic (16:0; 29.33 wt %) followed, in decreasing concentrations, by oleic ($18:1^{c9}$; 23.55 wt %), stearic (18:0; 11.92 wt %), and myristic (14:0; 10.15 wt %) acids.

We were able to identify 6, 8, 12, and 20 SNPs in GPAT4, AGPAT1, GPAT1, and LPIN1 genes, respectively (table 2.4) and to validate those SNPs using the Sequenom MassARRAY system. The majority of SNPs were intronic with a few synonymous and nonsynonymous mutations. The interesting finding was a relatively large number of nonsynonymous mutations (6 SNPs) discovered in the LPIN1 gene. After genotyping animals for the discovered SNPs, the PHASE program was used to reconstruct intragenic haplotypes. There were 6, 6, 5, and 7 haplotypes determined for GPAT4, GPAT1, AGPAT1, and LPIN1 genes, respectively, and their population frequencies were above 5 % (table 2.5).

F-tests were used to determine the overall haplotype effects on milk fatty acid composition for each gene separately (table 2.6). Results with p-value ≤ 0.0125 were considered to be significant, as determined by applying the Bonferroni adjustment to account for testing the association of genetic polymorphisms with milk fatty acid composition in each of four different genes. The overall haplotype effect of GPAT4 was associated significantly with atherogenic index (AI), SFA, UFA, MUFA, PUFA, SFA/UFA, caprylic (8:0), capric (10:0), lauric (12:0), myristic (14:0), palmitic (16:0),

oleic ($18:1^{c9}$) acids, CLA ($18:2^{c9, t11}$), C16 and C18 desaturation indices (table 2.6). There was a tendency for the association between the overall haplotype effect of GPAT4 and linoleic ($18:2^{c9, c12}$) acid concentration (p-value = 0.0126). The overall haplotype effect of GPAT1 was significantly associated with milk fat percentage, caproic (6:0), caprylic (8:0), capric (10:0), tridecylic (13:0), margaric (17:0), and myristoleic ($14:1^{c9}$) acid concentrations, and C14 desaturation index. The AGPAT1 gene showed significant associations of its haplotypes with PUFA, caproic (6:0), pentadecylic (15:0), margaric (17:0), myristoleic ($14:1^{c9}$), linoleic ($18:2^{c9, c12}$) acids, and CLA ($18:2^{c9, t11}$). The haplotypes from the LPIN1 gene affected only myristoleic ($14:1^{c9}$) acid concentration and C14 desaturation index.

Pairwise comparisons of the haplotype effects on milk fatty acid composition within GPAT4 (table 2.7) revealed that the haplotype h8 was the most desirable to select for animals producing milk with lower AI, lower concentrations of SFA and atherogenic fatty acids such as palmitic (16:0) and lauric (12:0) acids, lower concentration of capric (10:0) acid, lower SFA/UFA, and higher concentrations of UFA, MUFA, PUFA, and oleic ($18:1^{c9}$) acid (table 2.7). Thus, the haplotype h8 of GPAT4 was associated with 0.13 lower AI, 1.48 wt % lower SFA, 1.48 wt % higher UFA, 1.34 wt % higher MUFA, 0.12 lower SFA/UFA, 0.66 wt % lower palmitic (16:0) acid, and 0.80 wt % higher oleic ($18:1^{c9}$) acid concentrations compared with the effects of haplotype h6 of GPAT4. The haplotype h8 of GPAT4 also was associated with 0.21 wt % lower capric (10:0) acid and 0.23 wt % lower lauric (12:0) acid concentrations compared with the effects of haplotype h2 of GPAT4. Moreover, the haplotype h8 of GPAT4 was associated with 0.23 wt % higher PUFA concentration compared with the effects of haplotype h1 of GPAT4.

The second most desirable haplotypes of GPAT4 to select for animals producing healthier milk were haplotypes h10 and h3 that had similar effects on milk fatty acid composition in relation to each other, but the size of the their effects was smaller compared with the effects of haplotype h8 of GPAT4 (table 2.7). The haplotype h10 of GPAT4 was associated with 0.90 wt % lower SFA, 0.90 wt % higher UFA, 0.86 wt % higher MUFA concentrations, 0.061 lower SFA/UFA, and 0.048 higher C16 and 0.01 higher C18 desaturation indices compared with the effects of haplotype h6 of GPAT4. The haplotype h10 of GPAT4 also was associated with 0.18 wt % lower lauric (12:0) acid and 0.15 wt % higher PUFA concentrations compared with the effects of haplotypes h2 and h1 of GPAT4, respectively. The haplotype h3 of GPAT4 was associated with 0.90 wt % lower SFA, 0.90 wt % higher UFA, 0.86 higher MUFA concentrations, 0.069 wt % lower SFA/UFA, 0.54 wt % lower palmitic (16:0) acid, 0.63 wt % higher oleic ($18:2^{c9}$) acid concentrations, 0.043 higher C16 and 0.01 higher C18 desaturation indices compared with the effects of haplotype h6 of GPAT4. The haplotype h3 of GPAT4 also was associated 0.15 wt % higher PUFA concentration compare with the effects of haplotype h1 of GPAT4.

The haplotype h6 of GPAT4 was the most undesirable to select for animals producing healthier milk because, in addition to earlier mentioned negative effects of haplotype h6 compared with the effects of haplotypes h8, h10, and h3 of GPAT4, haplotype h6 of GPAT4 also was associated with 0.74 wt % lower MUFA, 0.61 wt % lower oleic ($18:1^{c9}$) acid concentrations, and 0.009 lower C18 index compared with the effects of haplotype h1 of GPAT4. The haplotype h6 of GPAT4 also was associated with 0.013 lower C18 desaturation index compared with the effects of haplotype h2 of GPAT4.

The haplotype h2 of GPAT1 was associated with lower percentage of milk fat, lower concentrations of short- and medium-chain SFA, and lower myristoleic (14:1) acid concentration compared with the effects of other haplotypes of GPAT1 (table 2.8). In particular, haplotype h2 of GPAT1 was associated with 0.086 wt % lower caproic (6:0) and 0.057 wt % lower caprylic (8:0) acid concentrations compared with the effects of the haplotype h8 of GPAT1. The haplotype h2 of GPAT1 also was associated with 0.056 wt % lower caprylic (8:0), 0.016 wt % lower tridecylic (13:0), and 0.066 wt % lower myristoleic ($14:1^{c9}$) acid concentrations compared with the effects of haplotype h12 of GPAT1 and 0.015 wt % lower tridecylic (13:0) acid concentration compared with the effects of haplotype h1 of GPAT1. Finally, haplotype h2 was associated with 0.15 % lower milk fat, 0.051 wt % lower caprylic (8:0), and 0.15 wt % lower capric (10:0) acid concentrations compared with the effects of haplotype h9 of GPAT1.

The haplotype h9 of GPAT1 was associated with higher percentage of milk fat, higher concentration of caprylic (8:0) and capric (10:0) acids, lower concentrations of myristoleic ($14:1^{c9}$) and margaric (17:0) acids, and lower C14 desaturation index compared with the effects of other haplotypes of GPAT1 (table 2.8). Thus, haplotype h9 of GPAT1 was associated with 0.11 % higher milk fat, 0.073 wt % lower myristoleic ($14:1^{c9}$), 0.013 wt % lower margaric (17:0) acid concentrations, and 0.0071 lower C14 desaturation index compared with the effects of haplotype h7 of GPAT1. The haplotype h9 also was associated with 0.065 and 0.10 wt % lower myristoleic ($14:1^{c9}$) acid concentration and 0.0058 and 0.0082 lower C14 desaturation index compared with the effects of the haplotypes h8 and h12 of GPAT1, respectively. The haplotype h9 of GPAT1 also was associated with 0.016 wt % lower margaric (17:0) acid concentration

compared with the effects of haplotype h12 of GPAT1. The haplotype h9 of GPAT1 was associated with 0.15 % higher milk fat, 0.051 wt % higher caprylic (8:0) and 0.15 wt % higher capric (10:0)) acid concentrations compared with the effects of haplotype h2 of GPAT1. The haplotype h12 of GPAT1 was associated with 0.072 wt % higher myristoleic (14:1) acid concentration and 0.0057 higher C14 desaturation index compared with the effects of haplotype h1 of GPAT1.

The haplotype h9 of AGPAT1 was associated with the lowest myristoleic ($14:1^{c9}$) acid concentration compared with the effects of the rest of the haplotypes of AGPAT1, and haplotype h10 of AGPAT1 was associated with the lowest PUFA, linoleic ($18:2^{c9, c12}$) acid and CLA ($18:2^{c9, t11}$) concentrations compared with the effects of the rest of the haplotypes of AGPAT1 (table 2.9). In particular, haplotype h9 of AGPAT1 was associated with 0.067, 0.084, 0.073, and 0.086 wt % lower myristoleic ($14:1^{c9}$) acid concentration compared with the effects of h2, h4, h6, and h10 haplotypes of AGPAT1, respectively. The haplotype h10 of AGPAT1 was associated with 0.16 wt % lower PUFA and 0.12 wt % lower linoleic ($18:2^{c9, c12}$) acid concentrations compared with the effects of the haplotype h6 of AGPAT1. The haplotype h10 also was associated with 0.12 wt % lower PUFA, 0.082 wt % lower linoleic ($18:2^{c9, c12}$) acid, 0.035 wt % lower CLA ($18:2^{c9, t11}$), and 0.045 wt % higher caproic (6:0) acid concentrations compared with the effects of haplotype h2 of AGPAT1. The haplotype h10 of AGPAT1 also was associated with 0.016 wt % lower margaric (17:0) acid concentration compared with the effects of haplotype h4 of AGPAT1. The haplotype h22 of LPIN1 was associated with the lowest concentration of myristoleic (14:1) acid and the lowest C14 desaturation index (table 2.9). Thus, the haplotype h22 of LPIN1 was associated with 0.085, 0.081, and 0.078 wt %

lower myristoleic ($14:1^{c9}$) acid concentration and 0.0073, 0.073, and 0.0067 lower C14 desaturation index compared with the effects of the h1, h24, and h26 haplotypes of LPIN1.

The allele substitution effect of DGAT1 A232K mutation (table 2.10) was associated with milk fat percentage, AI, concentrations of SFA, UFA, MUFA, PUFA, SFA/UFA, concentrations of palmitic (16:0), stearic (18:0) and other fatty acids. The significance was declared when $p \leq 0.05$. In particular, DGAT1 232K mutation was associated with 0.22 % higher milk fat, 0.10 higher AI, 1.05 wt % higher SFA, 1.05 wt % lower UFA, 0.88 wt % lower MUFA, 0.16 wt % lower PUFA concentrations, 0.086 higher SFA/UFA, 0.070 wt % higher lauric (12:0), 0.066 wt % higher myristoleic ($14:1^{c9}$), 0.87 wt % higher palmitic (16:0), 0.23 wt % lower stearic (18:0), 0.037 wt % lower oleic ($18:1^{c9}$), 0.038 wt % lower linoleic ($18:2^{c9, c12}$), 0.042 wt % lower CLA ($18:2^{c9, t11}$), 0.041 wt % higher caproic (6:0), 0.042 wt % higher caprylic (8:0), 0.086 wt % higher capric (10:0), 0.013 wt % higher tridecylic (13:0), 0.050 wt % higher pentadecylic (15:0) acid concentrations, 0.048 higher C14 desaturation index, and 0.015 lower elongation index compared with the effects of DGAT1 232A mutation. In addition, we tested FAS g. 17924G>A polymorphism for the association with milk fatty acid composition and the significance was declared when $p \leq 0.05$. Thus, the FAS g. 17924A allele was associated with 0.11 wt % higher myristic (14:0) and 0.17 wt % higher palmitic (16:0) acid concentrations.

Discussion

The overall effect of the GPAT4 haplotypes on milk fatty acid composition identified herein is in agreement with GPAT4 substrate specificities reported earlier [14]. The concentrations of GPAT4 substrates such as lauric (12:0), palmitic (16:0), oleic ($18:1^{c9}$), and linoleic ($18:2^{c9, c12}$) acids were affected by the GPAT4 haplotypes (table 2.6). In addition, AI, concentrations of SFA, UFA, MUFA, PUFA, SFA/UFA, C16 and C18 desaturation indices were affected by GPAT4 haplotypes as a result of the changes in the concentrations of the earlier mentioned fatty acids. The concentrations of fatty acids that are not typical GPAT4 substrates were affected by the GPAT4 haplotypes as well probably because of the alterations in the pool of fatty acids for TAG biosynthesis caused by the changes in the concentrations of the GPAT4 fatty acid substrates. The pairwise comparison of the GPAT4 haplotype effects on milk fatty acid composition showed that the haplotype h8 was the most desirable in terms of milk healthfulness followed by the haplotypes h10 and h3. The haplotype h6 of GPAT4 was the most undesirable to select for animals producing healthier milk. The effects of the haplotypes h1 and h2 of GPAT4 on milk healthfulness were in between the effects of the haplotype h6 and the haplotypes h3 and h10 of GPAT4, and thus those effects were not very desirable for improving milk healthfulness. The haplotype sequence comparison revealed a “tag” SNP that will allow distinguishing animals with the haplotype h8 of GPAT4 from the animals with any other GPAT4 haplotype. The “tag” SNP is intronic SNP6 located between exons 7 and 8 of GPAT4 and allele A is present in the haplotype h8 of GPAT4 (tables 2.4 and 2.5). There was no significant overall haplotype effect of GPAT4 on the percentage of milk fat (table

2.6), suggesting that it is possible to select for animals with the desirable fatty acid profile without affecting milk fat percentage.

The overall effect of GPAT1 haplotypes was associated with milk fat percentage, myristoleic ($14:1^{c9}$) acid concentration, and the concentrations of different SFA (table 2.6). Apart from milk fat and myristoleic ($14:1^{c9}$) acid, the affected SFA are the usual substrates of GPAT1 [14]. The importance of GPAT1 haplotype effects is related to their association with milk fat percentage. Even though the concentrations of different SFA were affected by GPAT1 haplotypes, none of the affected SFA is considered to be atherogenic and the total SFA concentration was not affected by GPAT1 haplotypes either. The pairwise comparison of the GPAT1 haplotype effects on milk fat percentage and milk fatty acid composition showed that the haplotype h9 of GPAAT1 always was associated with the higher milk fat percentage compared with the effects of the haplotypes h2 and h7 of GPAT1. We, however, were not able to identify a "tag" SNP for the haplotype h9 of GPAT1. The haplotype h2 of GPAT1 was associated with lower milk fat percentage compared with the effects of the haplotype h9 of GPAT1. A "tag" SNP for the haplotype h2 of GPAT1 was identified as SNP12, a nonsynonymous mutation located in exon 1 of GPAT1 that causes a substitution of argenine (Arg) by tryptophan (Trp) at 26 amino acid residue in GPAT1 polypeptide chain. The allele T of SNP12 was present in the haplotype h2 of GPAT1. In addition to the association with milk fat percentage, the haplotype h2 of GPAT1 was associated with low concentrations of mainly short- and medium-chain SFA. It is likely that the presence of Trp instead of Arg at position 26 in GPAT1 protein alters its substrate specificity, leading to decreased esterification of caproic (6:0), caprylic (8:0), capric (10:0), tridecylic (13:0) acids to sn-1 position of

glycerol-3-phsophate. The haplotype h7 of GPAT1 also was associated with lower milk fat percentage compared with the effect of haplotype h9 of GPAT1. We, however, were not able to identify any "tag" SNPs for the haplotype h7 of GPAT1.

The effects of AGPAT1 haplotypes on milk fatty acid composition were associated mainly with PUFA, linoleic ($18:2^{c9, c12}$) acid, CLA ($18:2^{c9, t11}$), and myristoleic ($14:1^{c9}$) acid concentrations (tables 2.6 and 2.9). The haplotype h10 of AGPAT1 was undesirable in terms of milk healthfulness because it always was associated with lower concentrations of PUFA, linoleic ($18:2^{c9, c12}$) acid and CLA ($18:2^{c9, t11}$) compared with the effects of haplotypes h2 and h6 of AGPAT1. The haplotype h9 of AGPAT1 always was associated with lower concentrations of myristoleic ($14:1^{c9}$) acid compared with the effects of haplotypes h2, h4, h6, and h10 of AGPAT1. The presence of the haplotype h9 of AGPAT1 could be considered undesirable when animals are selected for higher concentration of UFA in milk but, because of the small size of the haplotype effect on myristoleic ($14:1^{c9}$) acid concentration, the selection against the haplotype h9 of AGPAT1 might not produce the desirable results. The haplotype h6 of AGPAT1 is the most desirable to select for animals producing milk with higher concentrations of PUFA and linoleic ($18:2^{c9, c12}$) acid because the size of the effect of haplotype h6 of AGPAT1 on the concentrations of those fatty acids was very large (table 2.9). Another haplotype of AGPAT1 that can be used to select for animals producing milk with high concentrations of PUFA, linoleic ($18:2^{c9, c12}$) acid, and CLA ($18:2^{c9, t11}$) is haplotype h2. The sequence comparison among different AGPAT1 haplotypes revealed "tag" SNPs for the haplotypes h6, h9, and h10 of AGPAT1 (table 2.5). The "tag" SNPs for the haplotype h6 of AGPAT1 were intronic SNPs 37 and 38 located between exons 5 and 6. The "tag" SNPs

for the haplotype h9 of AGPAT1 were a synonymous SNP33 located in exon 3 and an intronic SNP34 located between exons 4 and 5. The "tag" SNPs for the haplotype h10 of AGPAT1 were intronic SNPs 32 and 35 located between exons 2 and 3 and exons 5 and 6, respectively.

The effects of LPIN1 haplotypes on milk fatty acid composition were associated with myristoleic ($14:1^{c9}$) acid concentration and C14 desaturation index (tables 2.6 and 2.9). The haplotype h22 of LPIN1 always was associated with lower concentration of myristoleic ($14:1^{c9}$) acid and lower C14 desaturation index compared with the effects of the haplotypes h1, h24, and h26 of LPIN1. The "tag" SNP for the haplotype h22 of LPIN1 was identified as SNP361, a nonsynonymous mutation that causes the substitution of asparagine by aspartic acid at 740 amino acid residue in a polypeptide chain of LPIN1. The allele A of SNP361 was present in the haplotype h22 of LPIN1. The selection against animals with the haplotype h22 of LPIN1 is desirable to increase the concentration of UFA in milk, but, because of the small size of the haplotype effect on myristoleic ($14:1^{c9}$) acid concentration, the selection goal might not be achieved.

We compared the results from our association study with the published data on a genome-wide association study that used 1,341 SNPs as genetic markers to genotype 849 Holstein dairy cows to identify quantitative trait loci (QTL) for milk fatty acids [38, 39]. The significant QTL for a number of medium- and long-chain fatty acids were reported to be between 27 and 31 cM on BTA26. Those QTL were attributed to the effects of polymorphisms in stearoyl-CoA desaturase-1 gene located around 16 cM on BTA26. The GPAT1 gene is located around 41 cM on BTA26, and the associations of the polymorphisms in GPAT1 gene with myristoleic (14:1) acid and C14 desaturation index

might be attributed to the QTL effects observed on BTA26 in the genome-wide study [39]. Even though the GPAT1 gene is not located in the exact area of observed QTL, the relatively low power to position those QTL correctly because of the limited number of genetic markers (34 markers on BTA26) and not large enough size of the phenotypic data can explain very wide confidence intervals for QTL and might not exclude the possibility that the observed effects either attributed to GPAT1 effects alone or to the combined effects of GPAT1 and SCD-1.

The QTL on BTA27 were associated only with butyric (4:0) and vaccenic ($18:1^{t11}$) acid concentrations, milk fat percentage, and total desaturation index, and those QTL were positioned around 34 cM, 44, 33, and 44 cM regions of the chromosome, respectively [38, 39]. The GPAT4 gene is located on BTA27 at 40 cM region, and, even though we did not calculate the total desaturation index, the haplotype effects of GPAT4 on C16 and C18 desaturation indices might be attributed to the QTL on BTA27 at 44 cM associated with the total desaturation index. Other haplotype effects of GPAT4 discovered in our study were not detected in the genome-wide association study because of the limited number of markers used (22 markers on BTA27) and not large enough phenotypic data and consequently, the low power to detect such effects.

The FAS g. 17924A>G polymorphism was associated with milk myristic (14:0) and palmitic (16:0) acid concentrations. In particular, the A allele was associated with higher concentration of myristic (14:0) acid and lower concentration of palmitic (16:0) acid. The same results were reported earlier only for milk myristic (14:0) acid and the SNPs that were associated with myristic (14:0) acid concentration in FAS were at positions 15531 and 15603, but not 17924 [36]. In a different study, the A allele of the

SNP at position 17924 was associated with higher concentration of myristic (14:0) acid [1] as it was in our study.

We performed a numerical comparison of the size of haplotype effects of GPAT4, GPAT1, AGPAT1, LPIN1 and the size of allelic effects of DGAT1 A232K and FAS g. 17924A>G on milk fat percentage and fatty acid composition to determine which genetic polymorphisms had the largest effect (table 2.11). The haplotype effect of GPAT4 was associated with at least the same or larger differences in AI (0.13), concentrations of SFA (1.48 wt %), UFA (1.48 wt %), MUFA (1.34 wt %), PUFA (0.23 wt %), SFA/UFA (0.12), and lauric (12:0; 0.23 wt %) acid concentration compared with the effects of DGAT1 A232K mutation. The haplotype effect of GPAT4 also was associated with at least the same or larger difference in capric (10:0; 0.21 wt %) acid concentration compared with the GPAT1 haplotype effects. The DGAT1 A232K mutation was associated with at least the same or larger difference in milk fat percentage (0.22 %) compared with the haplotype effect of GPAT1. The size of the effect of DGAT1 A232K mutation on the concentrations of palmitic (16:0; 0.87 wt %) and oleic ($18:1^{c9}$; 0.23 wt %) acids were at least the same or larger compared with the haplotype effect of GPAT4. The size of the effect of DGAT1 A232K mutation on the concentration of linoleic ($18:2^{c9, c12}$) acid was 0.12 wt % that was numerically similar to the AGPAT1 haplotype effect. The effects of GPAT1 haplotypes were associated with at least the same or larger concentrations of caproic (6:0), caprylic (8:0), tridecylic (13:0), myristoleic ($14:1^{c9}$) acid, and C14 desaturation index compare with the effects of DGAT1 A232K mutation.

Conclusions

The results of our study show that the polymorphisms in GPAT4 were associated with large differences in milk fatty acid composition. In particular, AI, concentrations of SFA, UFA, MUFA, PUFA, SFA/UFA, concentrations of capric (10:0), lauric (12:0), palmitic (16:0), oleic ($18:1^{c9}$) acids, CLA ($18:2^{c9, t11}$), C16 and C18 desaturation indices were affected the most by GPAT4 polymorphisms. The polymorphisms in GPAT4 provide a very valuable tool to improve milk healthfulness. Other polymorphisms discovered in our study were for milk fat percentage, concentrations of short- and medium-chain SFA, and myristoleic ($14:1^{c9}$) acid concentration in GPAT1, and for the concentrations of linoleic ($18:2^{c9, c12}$) acid and other UFA in AGPAT1. The polymorphisms in GPAT1 can be used to select for animals producing milk with higher percentage of fat and desirable concentrations of short- and medium-chain SFA. The polymorphisms in AGPAT1 can be used to select for animals producing milk with higher concentration of UFA and linoleic ($18:2^{c9, c12}$) acid, in particular.

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Table 2.1 Gene information

Gene	BTA	Chromosomal position, cM	Gene length, bp	Transcript length, bp	Protein length, aa	Number of exons	Strand direction
GPAT4	27	40	16752	2295	457	12	Forward
GPAT1	26	41	39943	6129	825	20	Reverse
AGPAT1	23	31	7481	2233	287	7	Forward
LPIN1	11	97	79574	3131	931	22	Reverse

AGPAT1, 1-acylglycerol-3-phosphate acyltransferase-1; BTA, *Bos taurus* autosome; GPAT1 and GPAT4, glycerol-3-phosphate acyltransferases-1 and -4; LPIN1, lipin-1

Table 2.2 Primer sequences and annealing temperatures for PCR

Gene	SNP	Forward primer	Reverse primer	PCR type[1]	Tm, °C
GPAT4	**3**	GCAGCAGGCAGGGAGTATG	GACAAGCACCCTTCCGAGAG	Regular	56
GPAT4	**4**	TGTCTTGGGACAGTGAGTGG	ATCTAAGAGCCTGTGGTTTGG	Regular	56
GPAT4	**5, 6**	GGGACTTTGTGTGAGGATGG	CGTAGGCTGCTATGACAATGG	Regular	56
GPAT4	**7**	CTCTCTCCAGCGTGTGTTCC	GTGGAGTGTCTTCCGAGCTG	Regular	56
GPAT4	**8**	GACACACGCACACAAGCAG	GTGTGGACGGAGTGAGCTG	Regular	56
AGPAT1	**32, 33**	GTGTGCAGGAGACACTGAGC	CTCCTGAGGGCAGCAGAG	Regular	60
AGPAT1	**34**	CCCAGTCTACCTCTTCCCTTATC	GGAAGACACACATGGAGCAG	Regular	56
AGPAT1	**35, 37, 38**	CAGAAGTCCCATGACAGCATC	AGGTGGACATCTCACCGTTC	Regular	56
AGPAT1	**40, 41**	TGTGCAAAGTCAGAGCATCC	TCCAAAGAGGAGAACAAGTGG	Regular	56
GPAT1	**25**	GTCTTTGTAAGGAACTCTTCTGTGAC	AGGCACTTGTCTTCCAGGAG	Regular	56
GPAT1	**23, 24**	CCTCCTACACTCTCTTCCTGTG	TTGAAACACATGGACACCTTC	Regular	56
GPAT1	**21, 22**	AACCTCAAATACCACGAAAGTCAC	CCATGGAGTGAGAGGTCAGG	Regular	58
GPAT1	**18**	CGTCGGAAATCTTAGTGCTTG	TCATCTTGGACATGACTCTCTCTC	Regular	56
GPAT1	**17**	TGCAGGCAGAATCTTCACC	CCTCTCAGCATGATCTTCCTTC	Regular	56
GPAT1	**16**	CATTCTATGAGTCATTCTGCACTTC	GGCACAGTCAGTTCCCAGTC	"Hot start"	59
GPAT1	**15**	CACCTTGAATTCTTGATTGCTC	CTCATCCTCCTCTCGCTCTC	"Hot start"	54
GPAT1	**14**	TTGCAAGATGCTGAGGAGTAG	AGAATAGTAATAGGTCTCTCAGGTTCC	"Hot start"	59
GPAT1	**12, 13**	GAAGTCCTCCTTAGTTCATTTGAC	AACTAACCTTCAAACAGCTGGAG	Regular	56
LPIN1	**369**	CACTTCTGCTCTGTCCTTCG	CTCCTCGGTCCTCTATGCTC	Regular	56
LPIN1	**364, 367**	CAGCAAAGTCACTGCTCCAG	GCTGTCCTTGCCTTCAGATG	Regular	56
LPIN1	**361, 362, 363**	CCATTGTGGTTTGTCACAGG	ACAAACCTGCTTGGAATTGG	"Hot start"	59
LPIN1	**360**	GGAAAGAACCATCCTCATCG	CCCAGAAACTGAACTACATGAGC	Regular	56
LPIN1	**359**	GCTCGATTGTAGACGACAGC	TGAGTAAATGACAGAGCACCAG	Regular	56
LPIN1	**355**	GAACGGTGCTCTGACCTGAC	GCTGCAACACCACCTTGG	Regular	56
LPIN1	**353, 354**	TTGACTCGACTCTTCTCTGACC	GAAGCGTTGGCTTTCTCAC	Regular	56
LPIN1	**352**	ATGTGGTCCTCTGCCATTG	TTTCACTTCCAAGAATCTATACGG	Regular	56

[1]"Hot start" PCR is a procedure that decreases non specific DNA amplification during the initial PCR cycles.

AGPAT1, 1-acylglycerol-3-phosphate acyltransferase-1; GPAT1 and GPAT4, glycerol-3-phosphate acyltransferases-1 and -4; LPIN1, lipin-1

Table 2.2 (continued)

Gene	SNP	Forward primer	Reverse primer	PCR type	Tm, °C
LPIN1	**350**	AGATAAGGAGAAATGGAAACTTGC	TGTGTTTAATCCTGATCTGTACCTTC	Regular	56
LPIN1	**346, 347, 348, 349**	ACCTGTGCTTGGACCTCTG	AACAGGACCATCTGAAACACTG	Regular	56
LPIN1	**340, 341**	CGTCTGAGCCACCTCTGG	CGGTGAACCAGAGCATCC	"Hot start"	59
LPIN1	**339**	TGGACAAGGAGAGAACATGG	CAGAGCAAACCACAGAAGGTC	Regular	56
DGAT1	**A232K**	TGGGCTCCGTGCTGGCCCTGATGGTCTA	TTGAGCTCGTAGCACAGGGTGGGGGCGA	"Hot start"	63

Table 2.3 Summary of milk fatty acid composition[1, 2]

Trait	Mean	Standard deviation	5% Quantile	95% Quantile
Milk production, kg/d	40.24	10.02	24.52	57.43
Milk fat, %	3.65	0.70	2.50	4.80
AI	2.07	0.46	1.27	2.80
SFA, wt %	63.93	4.47	55.57	70.29
UFA, wt %	36.07	4.47	29.71	44.43
MUFA, wt %	31.26	4.04	25.66	38.91
PUFA, wt %	4.81	0.92	3.46	6.42
SFA/UFA	1.81	0.34	1.25	2.37
4:0, wt %	2.47	0.76	1.29	3.80
6:0, wt %	1.55	0.35	0.90	2.05
8:0, wt %	1.00	0.23	0.57	1.35
10:0, wt %	2.47	0.63	1.35	3.47
12:0, wt %	2.94	0.73	1.61	4.10
13:0, wt %	0.18	0.08	0.07	0.33
14:0, wt %	10.15	1.60	6.80	12.26
14:1^{c9}, wt %	0.79	0.30	0.39	1.34
15:0, wt %	1.08	0.28	0.71	1.63
16:0, wt %	29.33	2.83	24.91	34.14
16:1^{c9}, wt %	1.75	0.41	1.16	2.47
17:0, wt %	0.63	0.09	0.50	0.77
18:0, wt %	11.92	2.37	8.44	16.14
18:2$^{c9, c12}$, wt %	3.24	0.70	2.23	4.47
18:1^{c9}, wt %	23.55	3.45	18.83	30.05
18:2^{c9,t11} (CLA), wt %	0.70	0.24	0.43	1.19
C14 Index[3]	0.07	0.02	0.04	0.11
C16 Index[4]	0.06	0.01	0.04	0.08
C18 Index[5]	0.66	0.05	0.59	0.74
Elongation index[6]	0.53	0.05	0.46	0.62

[1]Summary statistics were computed from 4397 milk samples collected from 551 cows.
[2]The data presented here are for only 16 fatty acids out of 39 fatty acids analyzed because the concentrations of the rest of fatty acids in milk were low.
[3]C14 Desaturation index = 14:1/ (14:0 + 14:1)
[4]C16 Desaturation index = 16:1/ (16:0 + 16:1)
[5]C18 Desaturation index = 18:1/ (18:0 + 18:1)
[6]Elongation index = (18:0 + 18:1)/ (16:0 + 16:1 + 18:0 + 18:1)

Table 2.4 SNPs used for the reconstruction of haplotypes in GPAT4, GPAT1, AGPAT1, and LPIN1 genes

SNP	Gene	Order in haplotype sequence	Nucleotide position, bp	Gene area	Alleles	Mutation type	A.A. Residue change
3	**GPAT4**	1	8435	Exon 3	T/C	Synonymous	None
4	**GPAT4**	2	9205	Intron 3-4	G/A	Intronic	None
5	**GPAT4**	3	11238	Intron 7-8	C/T	Intronic	None
6	**GPAT4**	4	11381	Intron 7-8	T/A	Intronic	None
7	**GPAT4**	5	12288	Intron 8-9	G/C	Intronic	None
8	**GPAT4**	6	15625	Intron 9-10	G/A	Intronic	None
32	**AGPAT1**	1	4660	Intron 2-3	T/G	Intronic	None
33	**AGPAT1**	2	4858	Exon 3	C/T	Synonymous	None
34	**AGPAT1**	3	5334	Intron 4-5	G/A	Intronic	None
35	**AGPAT1**	4	5567	Intron 5-6	G/A	Intronic	None
37	**AGPAT1**	5	5612	Intron 5-6	C/G	Intronic	None
38	**AGPAT1**	6	5662	Intron 5-6	C/T	Intronic	None
40	**AGPAT1**	7	6180	Intron 6-7	C/G	Intronic	None
41	**AGPAT1**	8	6354	Exon 7	A/G	Nonsynonymous	T267A
25	**GPAT1**	1	35991	Intron 19-20	A/G	Intronic	None
24	**GPAT1**	2	35680	Intron 19-20	G/A	Intronic	None
23	**GPAT1**	3	35483	Exon 19	T/C	Synonymous	None
21	**GPAT1**	4	34124	Exon 18	A/G	Synonymous	None
22	**GPAT1**	5	33921	Intron 17-18	A/G	Intronic	None
18	**GPAT1**	6	31956	Intron 16-17	C/T	Intronic	None
17	**GPAT1**	7	26680	Intron 12-13	C/T	Intronic	None
16	**GPAT1**	8	22884	Intron 10-11	G/A	Intronic	None
15	**GPAT1**	9	16972	Intron 7-8	A/G	Intronic	None
14	**GPAT1**	10	4694	Exon 3	G/A	Synonymous	None
12	**GPAT1**	11	105	Exon 1	C/T	Nonsynonymous	R26W
13	**GPAT1**	12	11	Exon 1	G/A	Synonymous	None
369	**LPIN1**	1	79397	Intron 21-22	C/T	Intronic	None
367	**LPIN1**	2	73986	Intron 20-21	G/A	Intronic	None
364	**LPIN1**	3	66001	Intron 18-19	G/A	Intronic	None
363	**LPIN1**	4	65969	Intron 18-19	G/A	Intronic	None
362	**LPIN1**	5	65917	Exon 18	C/T	Synonymous	None
361	**LPIN1**	6	65897	Exon 18	G/A	Nonsynonymous	D740N
360	**LPIN1**	7	65349	Intron 17-18	A/G	Intronic	None
359	**LPIN1**	8	58196	Intron 15-16	G/A	Intronic	None
355	**LPIN1**	9	48610	Intron 10-11	C/T	Intronic	None
354	**LPIN1**	10	46162	Exon 9	A/C	Nonsynonymous	H407P
353	**LPIN1**	11	46128	Exon 9	C/T	Nonsynonymous	P396S
352	**LPIN1**	12	40751	Intron 7-8	T/G	Intronic	None

AGPAT1, 1-acylglycerol-3-phosphate acyltransferase-1; GPAT1 and GPAT4, glycerol-3-phosphate acyltransferases-1 and -4; LPIN1, lipin-1

Table 2.4 (continued)

SNP	Gene	Order in haplotype sequence	Nucleotide position, bp	Gene area	Alleles	Mutation type	A.A. Residue change
350	**LPIN1**	13	38382	Exon 6	G/A	Nonsynonymous	R217H
349	**LPIN1**	14	34758	Exon 5	C/T	Synonymous	None
348	**LPIN1**	15	34714	Exon 5	A/G	Nonsynonymous	T125A
347	**LPIN1**	16	34643	Exon 5	T/C	Nonsynonymous	M101T
346	**LPIN1**	17	34607	Intron 4-5	C/T	Intronic	None
341	**LPIN1**	18	31071	Intron 3-4	G/C	Intronic	None
340	**LPIN1**	19	30751	Exon 3	G/A	Synonymous	None
339	**LPIN1**	20	-23	5' UTR	C/T	5’ UTR	None

Table 2.5 Intragenic haplotypes and their frequencies

Gene	Haplotype	Sequence[1, 2]	Frequency[3]
GPAT4	**h1**	$C_1G_2C_3T_4G_5G_6$	0.22
GPAT4	**h2**	$C_1G_2C_3T_4G_5A_6$	0.06
GPAT4	**h3**	$C_1G_2C_3T_4C_5G_6$	0.32
GPAT4	**h6**	$C_1G_2T_3T_4G_5G_6$	0.12
GPAT4	**h8**	$C_1G_2T_3\underline{\mathbf{A}}_4G_5G_6$	0.05
GPAT4	**h10**	$T_1G_2C_3T_4G_5G_6$	0.18
GPAT1	**h1**	$A_1G_2T_3A_4A_5C_6C_7G_8A_9G_{10}C_{11}G_{12}$	0.09
GPAT1	**h2**	$A_1G_2T_3A_4A_5C_6C_7G_8A_9G_{10}\underline{\mathbf{T}}_{11}G_{12}$	0.08
GPAT1	**h7**	$A_1A_2C_3G_4G_5T_6C_7G_8G_9G_{10}C_{11}G_{12}$	0.19
GPAT1	**h8**	$A_1A_2C_3G_4G_5T_6C_7G_8G_9A_{10}C_{11}G_{12}$	0.15
GPAT1	**h9**	$A_1A_2C_3G_4G_5C_6C_7G_8A_9G_{10}C_{11}G_{12}$	0.30
GPAT1	**h12**	$A_1A_2C_3G_4G_5C_6T_7G_8A_9G_{10}C_{11}G_{12}$	0.13
AGPAT1	**h2**	$G_1C_2G_3G_4C_5C_6C_7A_8$	0.54
AGPAT1	**h4**	$G_1C_2G_3G_4C_5C_6G_7G_8$	0.05
AGPAT1	**h6**	$G_1C_2G_3G_4\underline{\mathbf{G}}_5\underline{\mathbf{T}}_6C_7A_8$	0.13
AGPAT1	**h9**	$G_1\underline{\mathbf{T}}_2\underline{\mathbf{A}}_3G_4C_5C_6C_7A_8$	0.07
AGPAT1	**h10**	$\underline{\mathbf{T}}_1C_2G_3\underline{\mathbf{A}}_4C_5C_6C_7A_8$	0.18
LPIN1	**h1**	$C_1G_2A_3A_4T_5G_6A_7G_8C_9A_{10}C_{11}T_{12}G_{13}C_{14}A_{15}T_{16}C_{17}G_{18}G_{19}C_{20}$	0.14
LPIN1	**h4**	$C_1G_2A_3A_4T_5G_6A_7G_8C_9C_{10}C_{11}T_{12}G_{13}T_{14}G_{15}C_{16}C_{17}G_{18}G_{19}T_{20}$	0.13
LPIN1	**h13**	$C_1G_2A_3A_4C_5G_6G_7G_8C_9C_{10}C_{11}T_{12}G_{13}T_{14}G_{15}C_{16}T_{17}G_{18}G_{19}C_{20}$	0.09
LPIN1	**h18**	$C_1G_2G_3G_4C_5G_6A_7G_8C_9A_{10}C_{11}T_{12}G_{13}C_{14}A_{15}T_{16}C_{17}G_{18}G_{19}C_{20}$	0.12
LPIN1	**h22**	$C_1G_2G_3G_4C_5\underline{\mathbf{A}}_6A_7G_8T_9C_{10}T_{11}G_{12}A_{13}T_{14}G_{15}C_{16}T_{17}C_{18}G_{19}C_{20}$	0.07
LPIN1	**h24**	$C_1A_2G_3G_4C_5G_6A_7G_8T_9C_{10}T_{11}G_{12}A_{13}T_{14}G_{15}C_{16}T_{17}C_{18}G_{19}C_{20}$	0.10
LPIN1	**h26**	$T_1G_2G_3G_4C_5G_6A_7G_8T_9C_{10}T_{11}G_{12}A_{13}T_{14}G_{15}C_{16}T_{17}C_{18}G_{19}C_{20}$	0.15

[1]Subsripts indicate the order of SNPs in a particular haplotype for a particular gene
[2]The "tag" SNPs in the haplotypes of interest are highlighted in bold and underlined
[3]Only haplotypes with the population frequencies ≥ 5% were considered in the analysis
AGPAT1, 1-acylglycerol-3-phosphate acyltransferase-1; GPAT1 and GPAT4, glycerol-3-phosphate acyltransferases-1 and -4; LPIN1, lipin-1

Table 2.6 Results of the F-tests for the overall haplotype effect on milk fatty acid composition

Trait	GPAT4		GPAT1		AGPAT1		LPIN1	
	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value
Milk fat	0.40	0.88	3.49	0.0021*	2.14	0.059	2.60	0.11
AI	3.32	0.0031*	1.33	0.24	1.70	0.13	0.91	0.50
SFA	3.65	0.0014*	1.26	0.27	1.70	0.13	0.97	0.45
UFA	3.65	0.0014*	1.26	0.27	1.70	0.13	0.97	0.45
MUFA	3.89	0.0008*	1.57	0.15	1.10	0.36	1.10	0.36
PUFA	3.99	0.0006*	1.08	0.38	3.74	0.0023*	2.18	0.033
SFA/UFA	3.68	0.0013*	1.16	0.33	2.17	0.056	0.95	0.47
4:0	0.59	0.74	1.19	0.31	1.11	0.35	0.72	0.65
6:0	2.27	0.035	3.01	0.0065*	3.67	0.0027*	1.02	0.41
8:0	3.70	0.0012*	3.00	0.0067*	2.61	0.024	1.30	0.25
10:0	5.14	<.0001*	2.93	0.0078*	1.15	0.33	1.30	0.25
12:0	4.57	0.0001*	2.04	0.059	0.44	0.82	1.11	0.35
13:0	1.46	0.19	2.79	0.011*	1.76	0.12	0.65	0.71
14:0	3.18	0.0043*	2.04	0.059	0.50	0.77	0.61	0.74
14:1^{c9}	1.24	0.28	11.16	<.0001*	3.04	0.01*	3.66	0.0007*
15:0	0.67	0.68	0.76	0.60	3.01	0.011*	0.95	0.47
16:0	3.99	0.0006*	1.39	0.21	2.09	0.064	1.42	0.19
16:1^{c9}	2.07	0.054	2.43	0.025	2.57	0.026	0.22	0.98
17:0	0.64	0.70	4.96	<.0001*	5.50	<.0001*	1.88	0.070
18:0	1.75	0.11	1.68	0.12	1.38	0.23	0.43	0.88
18:1^{c9}	3.52	0.0019*	1.99	0.065	0.66	0.65	1.08	0.37
18:2$^{c9, c12}$	2.73	0.0126	1.18	0.31	3.05	0.0098*	2.53	0.014
18:2$^{c9, t11}$ (CLA)	2.89	0.0086*	1.23	0.29	3.51	0.0038*	0.72	0.66
Index 14	2.30	0.033	11.79	<.0001*	2.45	0.033	4.20	0.0001*
Index 16	3.07	0.0056*	2.49	0.022	1.48	0.19	0.26	0.97
Index 18	3.03	0.0062*	1.36	0.23	1.22	0.30	0.86	0.54
Elongation index	1.81	0.095	1.48	0.18	1.79	0.11	0.82	0.57

*The Bonferroni adjustment was used to account for multiple testing on four different genes. The significance of haplotype effects was declared if $p \leq 0.0125$ (0.05 divided by the number of genes that was 4)

AGPAT1, 1-acylglycerol-3-phosphate acyltransferase-1; GPAT1 and GPAT4, glycerol-3-phosphate acyltransferases-1 and -4; LPIN1, lipin-1

The numbers for the milk samples used in the analysis and the cows from which the samples were collected were the following: 3768 and 469; 3715 and 462; 3653 and 454; and 3986 and 501 for AGPAT1, GPAT1, GPAT4, and LPIN1, respectively.

Table 2.7 Pairwise comparisons of GPAT4 haplotype effects on milk fatty acid composition[1]

$H\text{-}H_b$	PUFA
H3-H1	0.15 (0.039) 0.0002
H8-H1	0.23 (0.063) 0.0003
H10-H1	0.15 (0.046) 0.0014

$H\text{-}H_b$	10:0	12:0
H8-H2	-0.21 (0.062) 0.0009	-0.23 (0.07) 0.0013
H10-H2	NS	-0.18 (0.055) 0.0017

$H\text{-}H_b$	AI	SFA	UFA	MUFA	SFA/ UFA	16:0	$18:1^{c9}$	C16 Index	C18 Index
H1-H6	NS	NS	NS	0.74 (0.24) 0.0023	NS	NS	0.61 (0.19) 0.0014	NS	0.009 (0.003) 0.0022
H2-H6	NS	NS	NS	NS	NS	NS	NS	NS	0.013 (0.004) 0.0007
H3-H6	NS	-0.90 (0.25) 0.0004	0.90 (0.25) 0.0004	0.86 (0.23) 0.0002	-0.069 (0.019) 0.0003	-0.54 (0.15) 0.0003	0.63 (0.18) 0.0006	0.0026 (0.0008) 0.0019	0.01 (0.003) 0.0004
H8-H6	-0.13 (0.036) 0.0002	-1.48 (0.38) <.0001	1.48 (0.38) <.0001	1.34 (0.34) <.0001	-0.12 (0.028) <.0001	-0.66 (0.22) 0.0031	0.80 (0.27) 0.0029	NS	NS
H10-H6	NS	-0.90 (0.27) 0.0009	0.90 (0.27) 0.0009	0.86 (0.25) 0.0005	-0.061 (0.02) 0.0027	NS	NS	0.0032 (0.0009) 0.0003	0.01 (0.003) 0.0012

[1]The first number out of three numbers in a cell is an estimate of the haplotype substitution effect expressed in wt % for all individual fatty acids and fatty acid groups excluding indices. The second number in the parenthesis is a standard error of an estimate of the haplotype substitution effect expressed in the same units as the estimate. The third number is a p-value.

*The Bonferroni adjustment for multiple comparisons was used, and significance was declared if p-value was ≤ 0.0033 (0.05 divided by the number of comparisons that was 15.

$H\text{-}H_b$, indicates a pair of haplotypes for which the haplotype effects on milk fatty acid composition were compared with H_b, indicating the position of a haplotype used as a baseline.

GPAT4, glycerol-3-phosphate acyltransferases-4; NS, not significant

The numbers for the milk samples used in the analysis and the cows from which the samples were collected were 3653 and 454.

Table 2.8 Pairwise comparisons of GPAT1 haplotype effects on milk fatty acid composition[1]

H-H_b	13:0	14:1^{c9}	C14 Index
H2-H1	-0.015 (0.005) 0.0024	NS	NS
H12-H1	NS	0.072 (0.020) 0.0004	0.0057 (0.0017) 0.0009

H-H_b	6:0	8:0	13:0	14:1^{c9}
H8-H2	0.086 (0.025) 0.0007	0.057 (0.018) 0.0014	NS	NS
H12-H2	NS	0.056 (0.018) 0.0017	0.016 (0.004) 0.0004	0.066 (0.023) 0.0033

H-H_b	Milk fat	8:0	10:0	14:1^{c9}	17:0	C14 Index
H2-H9	-0.15 (0.047) 0.0019	-0.051 (0.016) 0.0014	-0.15 (0.044) 0.0008	NS	NS	NS
H7-H9	-0.11 (0.033) 0.0015	NS	NS	0.073 (0.014) <.0001	0.013 (0.0032) <.0001	0.0071 (0.0012) <.0001
H8-H9	NS	NS	NS	0.065 (0.016) <.0001	NS	0.0058 (0.0013) <.0001
H12-H9	NS	NS	NS	0.10 (0.016) <.0001	0.016 (0.0036) <.0001	0.0082 (0.0013) <.0001

[1]The first number out of three numbers in a cell is an estimate of the haplotype substitution effect expressed in percentages for milk fat, wt % for all individual fatty acids and fatty acid groups excluding indices. The second number in the parenthesis is a standard error of an estimate of the haplotype substitution effect expressed in the same units as the estimate. The third number is a p-value.

*The Bonferroni adjustment for multiple comparisons was used, and significance was declared if p-value was ≤ 0.0033 (0.05 divided by the number of comparisons that was 15.

H-H_b, indicates a pair of haplotypes for which the haplotype effects on milk fatty acid composition were compared with H_b, indicating the position of a haplotype used as a baseline.

GPAT1, glycerol-3-phosphate acyltransferases-1; NS, not significant

The numbers for the milk samples used in the analysis and the cows from which the samples were collected were 3715 and 462.

Table 2.9 Pairwise comparisons of AGPAT1 and LPIN1 haplotype effects on milk fatty acid composition[1]

AGPAT1		LPIN1		
H-H_b	**14:1^{c9}**	**H-H_b**	**14:1^{c9}**	**C14 Index**
H2-H9	0.067 (0.020) 0.0007	**H1-H22**	0.085 (0.023) 0.0002	0.0073 (0.0020) 0.0002
H4-H9	0.084 (0.028) 0.0029	**H24-H22**	0.081 (0.024) 0.001	0.0073 (0.0021) 0.0005
H6-H9	0.073 (0.023) 0.002	**H26-H22**	0.078 (0.022) 0.0003	0.0067 (0.0018) 0.0003
H10-H9	0.086 (0.023) 0.0002			

AGPAT1					
H-H_b	**PUFA**	**18:2$^{c9, c12}$**	**18:2$^{c9, t11}$ (CLA)**	**6:0**	**17:0**
H2-H10	0.12 (0.036) 0.0008	0.082 (0.026) 0.0017	0.035 (0.012) 0.0044	-0.045 (0.015) 0.0023	NS
H4-H10	NS	NS	NS	NS	0.016 (0.0053) 0.0023
H6-H10	0.16 (0.049) 0.0013	0.12 (0.035) 0.0008	NS	NS	NS

[1]The first number out of three numbers in a cell is an estimate of the haplotype substitution effect expressed in wt % for all individual fatty acids and fatty acid groups excluding indices. The second number in the parenthesis is a standard error of an estimate of the haplotype substitution effect expressed in the same units as the estimate. The third number is a p-value.

*The Bonferroni adjustment for multiple comparisons was used, and significance was declared if p-value were ≤ 0.005 and 0.0024 for AGPAT1 and LPIN1, respectively (0.05 divided by the number of comparisons that were 10 and 21 for AGPAT1 and LPIN1, respectively).

H-H_b, indicates a pair of haplotypes for which the haplotype effects on milk fatty acid composition were compared with H_b, indicating the position of a haplotype used as a baseline.

AGPAT1, 1-acylglycerol-3-phosphate acyltransferase-1; LPIN1, lipin-1; NS, not significant

The numbers for the milk samples used in the analysis and the cows from which the samples were collected were the following: 3768 and 469; and 3986 and 501 for AGPAT1 and LPIN1, respectively.

Table 2.10 Allele substitution effects of DGAT1 A2232K and FAS g. 17924G>A polymorphisms on milk fatty acid composition

Trait	DGAT1 (A232K)			FAS (g. 17924G>A)		
	Estimate	Standard error	p-value	Estimate	Standard error	p-value
Milk fat, %	0.22	0.030	<.0001	0.0080	0.023	0.72
AI	0.10	0.019	<.0001	0.008	0.014	0.58
SFA, wt %	1.05	0.20	<.0001	-0.099	0.14	0.50
UFA, wt %	-1.05	0.20	<.0001	0.099	0.14	0.50
MUFA, wt %	-0.88	0.19	<.0001	0.11	0.13	0.41
PUFA, wt %	-0.16	0.037	<.0001	-0.011	0.028	0.70
SFA/ UFA	0.086	0.015	<.0001	-0.004	0.011	0.69
4:0, wt %	0.0046	0.025	0.86	-0.024	0.020	0.22
6:0, wt %	0.060	0.015	0.0001	-0.015	0.011	0.17
8:0, wt %	0.042	0.011	<.0001	-0.009	0.008	0.25
10:0, wt %	0.086	0.030	0.0042	-0.013	0.022	0.57
12:0, wt %	0.070	0.033	0.035	-0.003	0.024	0.90
13:0, wt %	0.013	0.003	<.0001	0.000	0.002	0.85
14:0, wt %	0.11	0.061	0.081	0.11	0.047	0.018
14:1^{c9}, wt %	0.042	0.015	0.0037	0.0012	0.0098	0.90
15:0, wt %	0.056	0.012	<.0001	-0.0042	0.0086	0.63
16:0, wt %	0.87	0.11	<.0001	-0.17	0.085	0.042
16:1^{c9}, wt %	0.087	0.022	<.0001	-0.0038	0.016	0.81
17:0, wt %	-0.0035	0.0032	0.27	0.0016	0.0023	0.49
18:0, wt %	-0.23	0.11	0.032	0.040	0.078	0.61
18:1^{c9}, wt %	-0.88	0.14	<.0001	0.036	0.10	0.73
18:2$^{c9, c12}$, wt %	-0.12	0.026	<.0001	-0.0077	0.020	0.70
18:2$^{c9, t11}$ (CLA), wt %	-0.028	0.013	0.028	0.0061	0.0093	0.51
C14 Index	0.0030	0.0013	0.016	-0.00022	0.00083	0.79
C16 Index	0.00099	0.00068	0.14	0.00024	0.00049	0.63
C18 Index	-0.004	0.002	0.062	0.000	0.002	0.83
Elongation index	-0.015	0.002	<.0001	0.002	0.001	0.23

DGAT1, diacylglycerol acyltransferase-1; FAS, fatty acid synthase

The numbers for the milk samples used in the analysis and the cows from which the samples were collected were the following: 2587 and 325; and 4003 and 500 for DGAT1 and FAS, respectively.

Table 2.11 Summary of the largest effects of polymorphisms in GPAT4, GPAT1, AGPAT1, LPIN1, DGAT1, and FAS genes on milk fat percentage and milk fatty acid composition

Trait	Gene					
	GPAT4	**GPAT1**	**AGPAT1**	**LPIN1**	**DGAT1**	**FAS**
Milk fat, %	NS	0.15	NS	NS	0.22	NS
AI	0.13	NS	NS	NS	0.10	NS
SFA, wt %	1.48	NS	NS	NS	1.05	NS
UFA, wt %	1.48	NS	NS	NS	1.05	NS
MUFA, wt %	1.34	NS	NS	NS	0.88	NS
PUFA, wt %	0.23	NS	NS	NS	0.16	NS
SFA/ UFA	0.12	NS	NS	NS	0.086	NS
4:0, wt %	NS	NS	NS	NS	NS	NS
6:0, wt %	NS	0.086	0.045	NS	0.060	NS
8:0, wt %	NS	0.057	NS	NS	0.042	NS
10:0, wt %	0.21	0.15	NS	NS	0.086	NS
12:0, wt %	0.23	NS	NS	NS	0.070	NS
13:0, wt %	NS	0.016	NS	NS	0.013	NS
14:0, wt %	NS	NS	NS	NS	NS	0.11
$14:1^{c9}$, wt %	NS	0.10	0.086	0.085	0.042	NS
15:0, wt %	NS	NS	NS	NS	0.056	NS
16:0, wt %	0.66	NS	NS	NS	0.87	0.17
$16:1^{c9}$, wt %	NS	NS	NS	NS	0.087	NS
17:0, wt %	NS	0.016	0.016	NS	NS	NS
18:0, wt %	NS	NS	NS	NS	0.23	NS
$18:1^{c9}$, wt %	0.80	NS	NS	NS	0.88	NS
$18:2^{c9, c12}$, wt %	NS	NS	0.12	NS	0.12	NS
$18:2^{c9, t11}$ (CLA), wt %	NS	NS	0.035	NS	0.028	NS
C14 Index	NS	0.0082	NS	0.0073	0.0030	NS
C16 Index	0.0032	NS	NS	NS	NS	NS
C18 Index	0.013	NS	NS	NS	NS	NS
Elongation index	NS	NS	NS	NS	0.015	NS

AGPAT1, 1-acylglycerol-3-phosphate acyltransferase-1; FAS, fatty acid synthase; DGAT1, diacylglycerol acyltransferase-1; GPAT1 and GPAT4, glycerol-3-phosphate acyltransferases-1 and -4; LPIN1, lipin-1; NS not significant

CHAPTER 3. EFFECTS OF POLYMORPHISMS IN FABP3, FABP4, AND SLC27A6 GENES ON BOVINE MILK FATTY ACID COMPOSITION

A paper to be submitted to Physiological Genomics

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Abstract

The main goal of our study was to develop tools for the genetic selection of animals producing milk with lower concentration of saturated fatty acids (SFA) as a class and lower concentrations of individual atherogenic SFA, such as palmitic (16:0) and myristic (14:0), with the purpose of improving the healthfulness of bovine milk. We hypothesized that genetic polymorphisms in solute carrier family 27, isoform A6 (SLC27A6) fatty acid transport protein and fatty acid binding proteins-3 and -4 (FABP3 and FABP4) will affect the selectivity of fatty acid uptake into and fatty acid redistribution inside mammary epithelial cells, resulting in altered fatty acid composition of bovine milk. The objectives of our study were to discover genetic polymorphisms in SLC27A6, FABP3, and FABP4 and to test those polymorphisms for the association with milk fat percentage and fatty acid composition. The results of our study showed that the overall haplotype effect of SLC27A6 was associated significantly with the percentage of milk fat, the concentrations

of SFA, unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), SFA/UFA, and the concentrations of capric (10:0), lauric (12:0), myristic (14:0), and palmitic (16:0) acids in milk. The overall haplotype effect of FABP4 was associated significantly with the concentrations of SFA, UFA, MUFA, PUFA, SFA/UFA, the concentrations of linoleic ($18:2^{c9, c12}$) acid, conjugated linoleic acid ($18:2^{c9, t11}$), and C18 desaturation index. The overall haplotype effect of FABP3 was associated significantly with the concentrations of only pentadecylic (15:0) acid and elongation index. In conclusion, the results of our study show that the information about polymorphisms in SCL27A6, FABP3, and FABP4 can be used to select for cattle producing milk with healthier fatty acid composition and with higher percentage of milk fat.

Introduction

Dietary saturated fatty acids (SFA) are known for their adverse effects on plasma cholesterol concentrations in humans (32). For example, a 1% increase in dietary total energy from SFA will result in a 1.9 mg/dL increase in plasma total cholesterol (TC) and a 1.8 mg/dL increase in plasma low-density lipoprotein-cholesterol (LDL-C) concentrations (28). Particular attention also should be paid to the concentrations of palmitic (16:0) and myristic (14:0) acids in human diets because those fatty acids raise plasma TC and LDL-C concentrations much higher compared with other individual SFA (32). Bovine milk contains a relatively high percentage of SFA, with palmitic (16:0) acid being the most abundant among all fatty acids. The main objective of our study was to develop genetic markers allowing the selection of animals producing healthier milk with high concentration of UFA and low concentrations of SFA and palmitic (16:0) and

myristic (14:0) acids. The animal genetic approach to improve the healthfulness of milk was chosen over the nutritional regulation because of the moderate to high heritabilities of milk fatty acids (2, 8, 37, 47, 52) and the low conversion rate of dietary UFA into milk UFA as a consequence of ruminal biohydrogenation of UFA (18).

The uptake of long-chain fatty acids from plasma into a cell is mediated by fatty acid transport proteins that also are called solute carrier family 27 (SLC27) proteins (11, 17, 49). There are six members of SLC27 protein family (SLC27A1-6). The SLC27A1 is highly expressed in adipose tissue and skeletal and heart muscles, and its translocation from inside a cell to plasma membrane is stimulated by insulin. The SLC27A3 expression is not well established, but SLC27A4 is predominantly expressed in small intestine, adipose tissue, liver, heart, and skin. The SLC27A5 is abundant in liver and plays the major role in fatty acid uptake by that organ, SLC27A2 is expressed in liver and kidney, and SLC27A6 is mainly expressed in heart tissue. The information regarding the function of SLC27 protein family members in the mammary gland is limited. The expression of three members of SLC27 family, however, was detected in the mouse mammary gland. Those proteins were SLC27A1, SLC27A3, and SLC27A4, with SLC27A3 being the most abundant (41). In bovine mammary epithelial cells, the SLC27A6 was the major isoform to be expressed, followed by SLC27A1 and SLC27A5 (6, 7). The expression of all three SLC27 isoforms was highly up-regulated with the onset of lactation.

After entering a cell, fatty acids have to be either esterified to coenzyme A with a subsequent binding to acyl-CoA binding proteins or bound to fatty acid binding proteins (FABPs) as non-esterified fatty acids. The FABPs are 14-15 kDa proteins that reversibly bind non-esterified saturated and unsaturated long-chain fatty acids, eicosanoids, and

other lipids with the purpose of transporting or storing them inside a cell (20). Fatty acids esterified to coenzyme A can be bound by FABPs as well. There are nine members of the FABP protein family that are expressed differentially in multiple tissues. The most abundant isoforms of FABPs expressed in the lactating bovine mammary gland are FABP3, FABP4, and FABP5 (6). The expression of FABP3 was detected also at very high levels in mouse mammary gland (41). The FABP3 protein, also known as heart FABP, is expressed in tissues such as heart, skeletal muscle, mammary gland, brown adipose tissue, and others (20). The main function of FABP3 in heart and skeletal muscles is to channel fatty acids inside a cell towards mitochondrial β-oxidation. The discovery of FABP3 protein in mammary gland was related to the identification of a mammary-derived growth inhibitor (MDGI) that turned out to be a mixture of FABP3 and FABP4 proteins (9, 48). The MDGI inhibited the growth of human breast cancer cells (29). The FABP3 protein is highly expressed in the mammary gland during cell differentiation and formation of ductal structures at the onset of lactation (5), but the requirement of FABP3 for mammary tissue development and function is not well established (4, 15).

The FABP4, also known as adipocyte FABP, is highly expressed in mature and differentiating adipose tissues, and its expression is tightly regulated by fatty acids, PPAR-γ agonists, and insulin (20). There is not much information about FABP4 function in the mammary gland. The polymorphisms in FABP3 and FABP4 genes were studied in Korean native beef cattle in relation to carcass weight and back fat thickness (12). There was a significant association between back fat thickness and a few polymorphisms in the FABP4 gene. Considering the fact that FABP3, FABP4, and SLC27A6 are highly

expressed in bovine mammary gland during lactation with FABP3 being the second most abundant transcript among all measured (6, 7), we hypothesize that polymorphisms in SLC27A6, FABP3, and FABP4 genes can affect the selectivity of fatty acid uptake from the blood and fatty acid transport inside the mammary epithelial cells, resulting in differences in milk fatty acid composition.

Materials and methods

Milk fatty acid analysis

Milk samples were collected during the morning milking once a month throughout a 305-d lactation period and stored at -20 °C until further analysis. The extraction of milk fatty acids with subsequent esterification into methyl esters for the analysis by gas chromatography (GC) was performed according to the procedure published by Chouinard et al. (16) with minor modifications. The procedure is based on the original milk lipid extraction method with hexane and isopropanol developed by Hara and Radin (25) and fatty acid methyl ester production method developed by Christie (13). After thawing, milk samples were vortexed for 10 s, and 25 mL aliquots were transferred to 50 mL teflon centrifuge tubes pre-rinsed with chloroform/ methanol (2/1 vol.vol). Following the centrifugation at 17,800 × *g* for 30 min at 4 °C, the liquid phase of the milk samples was removed and 18 mL of hexane/isopropanol (3/2 vol/vol) were added for every gram of lipids with subsequent vortexing for 1 min. To achieve a better separation of hexane phase containing lipids from water phase containing proteins, 12 mL of 6.7 % sodium sulfate (Na_2SO_4) solution were added for every gram of lipids and samples were vortexed for 1 min. After the centrifugation at 2,500 × *g* for 5 min at 4 °C, the hexane fraction

containing lipids was removed into a scintillation vial pre-rinsed with chloroform/ methanol (2/1 vol/vol) and an aliquot containing 40 mg of lipids (400 μL) was transferred into an esterification vial . After the evaporation of hexane under nitrogen gas, 2 mL of hexane were added to the esterification vial followed by 40 μL of methyl acetate and vortexing for 30 s. The methylation step was initiated by the addition of 40 μL of sodium methoxide (0.4 mL of 5.4 M sodium methoxide in 1.75 mL of methanol) solution. The mixture was vortexed for 30 s and allowed to react for 10 min at room temperature. The reaction was terminated by the addition of 60 μL of oxalic acid (1 gram of oxalic acid/30 mL of diethyl ether) solution. The samples were centrifuged at 2,400 × *g* for 5 min at 4 °C, and a clear hexane fraction, which contained fatty acid methyl esters, was placed into GC vials, purged with nitrogen gas for 3 s, capped, and used for chromatographic analysis. In the above steps, capping of the vials that contained hexane lipid extract was preceded by purging with nitrogen gas for 3 s.

A Varian 3900 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA) equipped with CP-8400 auto-sampler, CP-8410 auto-injector, CP-1177 split/splitless injector, and flame ionization (FID) detector was used to analyze fatty acid methyl esters with helium as a carrier gas. One μL of fatty acid methyl esters in hexane was injected (split ratio 50:1) into a fused silica capillary column (Supelco^{TM}-2560 Capillary Column, 100 m x 0.25 mm i.d., with 0.2 μm film thickness) with the initial column temperature set at 70 °C and held for 4 min. Then, the column temperature was increased to 175 °C at the rate of 13 °C/min and held for 27 min with subsequent increase to 215 °C at the rate of 4 °C/min where it was held for 28 min (46). The injector and detector temperatures were set constant at 220 °C. Peak area was measured by integration using Star

Chromatography Workstation Version 6, and peaks were identified by comparing the retention times with separately run fatty acid methyl ester standards (Matreya LLC, Pleasant Gap, PA).

DNA extraction

Genomic DNA was extracted from 20 mL of blood obtained from the coccygeal vein of every dairy cow. The blood was collected into 10 mL EDTA Vacutainer tubes and kept at 4 °C. After transferring 20 mL of blood into 50 mL centrifuge tubes, the ice-cold 1X red blood cell (RBC) lysis buffer was added to fill in the tubes that were inverted and incubated on ice for 30 min. The samples were centrifuged at 300 × *g* for 5 min at 4 °C. Supernatant was aspirated and 20 mL of ice-cold 1X RBC lysis buffer were added again followed by a light vortexing and 30 min and incubation on ice. After centrifugation and aspiration steps performed as mentioned earlier, 20 mL of room temperature PBS buffer were added to wash the pellet and the centrifugation step was repeated again. Then, supernatant was discarded and pellet was resuspended in PBS, transferred to cryovial, and stored at -70 °C until further analysis. The DNA purification from the white blood cells was performed with DNeasy® Blood and Tissue Kit (Qiagen Inc., Valencia, CA) according to the instructions. The DNA concentration was measured using NanoDrop® ND1000, and dilutions were made with DNase-free water to desired concentration.

SNP discovery and genotyping

The SLC27A6, FABP3, and FABP4 genes (table 3.1), were sequenced in exonic and some intronic regions to discover SNPs. A set of 12 DNA samples from cows that were

daughters of 12 different sires was used for the SNP discovery. The PCR primers were designed with Primer3 (version 0.4.0) software (40). Conventional or "Hot-Start" PCR was performed to amplify the DNA regions of interest. For the regular PCR, the reaction mixture contained 50 ng of genomic DNA, 13.6 μL of DNA grade water, 2 μL of DMSO, 1.5 μL of $MgCl_2$ (25 mM), 0.1 μL of forward and reverse primers (100 ng/μL), 0.5 μL of dNTP mix (40 mM), 5 μL of 5X buffer, and 0.2 μL of Tag DNA polymerase (5 U/μL) with the total volume of the reaction mixture equal to 25 μL. For the HotStarTag® PCR, the reaction mixture was almost the same as for the regular PCR with the only difference that 5 μL of 10X buffer was used instead of 5X buffer and 0.2 μL of HotStarTag DNA polymerase (5 U/μL) was used instead of Tag DNA polymerase. The PCRs were performed in a DNA Engine thermal cycler (Bio-Rad) with the separate temperature cycle programs for the regular and HotStarTag® PCRs. The regular PCR was performed with the following program: 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, an optimal annealing temperature for a particular primer set (table 3.1) for 30 s, and 72 °C for 30 s with a final extension step at 72 °C for 10 min. The HotStarTag® PCR was performed with the following program: 95 °C for 15 min followed by 30 cycles of 94 °C for 1 min, an optimal annealing temperature for a particular primer set (table 3.1) for 1 min, and 72 °C for 1 min with a final extension step at 72 °C for 10 min. The optimal annealing temperature for a particular primer set was determined by a PCR temperature gradient. Before sequencing, the PCR product was cleaned up from unused primers and dNTPs with ExoSAP-IT® (USB Corporation, Cleveland, OH) that contained exonuclease 1 and shrimp alkaline phosphatase (24). Reverse and forward sequences representing 12 different samples were aligned using Vector NTI Advance™ 10 to identify SNPs.

Genotyping of discovered SNPs was performed with Sequenom MassARRAY platform using 10 ng of genomic DNA dissolved in DNase-free water (21). Haplotypes and their frequencies were estimated using PHASE (version 2.1) program (50, 51). Only samples with probability ≥0.9 for the best haplotype pair were used in the analysis. The restriction fragment length polymorphism technique was used to genotype animals for DGAT1 A232K mutation. A set of primers (table 3.2), was used to amplify a 405-bp region of DNA containing the mutation site by using a "Hot start" PCR. The resulting PCR product, then, was digested with EaeI restriction enzyme and run on a 2% agarose gel. There were the 230-bp and 175-bp DNA fragments on a gel when an animal had DGAT1 232K genotype and a 405-bp DNA fragment on a gel when an animal had DGAT1 232A genotype.

Statistical analysis

A linear mixed model for longitudinal data was used to analyze the data with PROC MIXED procedure of SAS 9.1 (2002). The haplotype substitution model (31) used to test the association between intragenic haplotypes and milk fatty acid composition was the following:

$Y = \mu + dim + dim^2 + dim^3 + cg + dim*cg + dim^2*cg + dim^3*cg + \sum b_k H_k + sire + \varepsilon$

where y is the response variable; μ is the mean response at 0 dim for a cow with no copies of the haplotypes; dim, dim^2, and dim^3 are covariates describing the effects of days in milk on milk fatty acid composition; cg is a fixed effect of the contemporary group (cg=8 classes); H_k is a haplotype effect fitted as a covariate and coded as 0, 1, or 2 for 0, 1, or 2 copies of each haplotype present in an animal; b_k is the partial regression

coefficient, which corresponds to the haplotype substitution effect for the k^{th} haplotype as a deviation from the effect of the most frequent haplotype that is set to zero to have a full rank model; sire is a sire random effect; and ε is a residual error. The higher order terms for days in milk were introduced to account for a non-linear trend of the response variables over time. The cubic term of days in milk allows the response variable to have different slopes whenever the direction of its trend changes over time. When the higher order terms for days in milk were not significant, they were dropped from the model. Contemporary groups were created by combining herd (two herds) and season of calving (four seasons: December to February, March to May, June to August, and September to November) effects. The year of sampling was not included in the model to avoid redundancy because the season of calving is already containing some information about the year when a particular sample was collected. Haplotypes with a population frequency more than 0.05 were used in the analysis, and those with frequency less than 0.05 were pooled in the "other" category. The CONTRAST statement in PROC MIXED was used to construct the F-tests of the overall haplotype effects, and, when the test was significant after applying the Bonferroni adjustment to account for multiple testing, the pairwise comparisons between all haplotypes within each gene were performed using the ESTIMATE statement and the estimates of the differences between haplotype effects, their standard errors, and p-values were reported. The REPEATED statement was used to indicate that milk samples were collected repeatedly for the same animal throughout the 305 d lactation period. Correlations between repeated measures taken on the same animal were modeled using a first order autoregressive covariance structure that assumes equal variances and correlations that decline exponentially with an increase in the distance

between time points (33). The covariance structure was selected on the basis of the biology of the experiment and using Akaike's and Bayesian Information Criteria and residual log likelihood (42).

For the traits that were significantly associated with the overall haplotype effect, we performed pairwise comparisons between different haplotypes within the same gene to determine which haplotypes had significant effects on differences in milk fatty acid composition compared with the effects of other haplotypes. The Bonferroni adjustment was used to control type I error rate during multiple comparisons. Bonferroni-adjusted significance levels were determined by dividing desired experiment-wide significance level (0.05) by the number of pairwise comparisons. The numbers of pairwise comparisons for SLC27A6, FABP3, and FABP4 genes were 10, 3, and 3, respectively that resulted in Bonferroni-adjusted significance levels of 0.005, 0.0167, and 0.0167, respectively. We compared haplotype sequences within each gene based on their effects on milk fatty acid composition to determine "tag" SNPs that can be used as genetic markers.

In recent years, a number of studies looked at the effects of SNPs from different genes on milk fatty acid composition (38, 43-45). It is difficult, however, to compare the size of the effects of different polymorphisms on any particular fatty acid concentration in milk because of the different approaches used to analyze the data. To compare the results of our study with earlier published data, we genotyped our animals for DGAT1 K232A and fatty acid synthase g. 17924A>G polymorphisms and analyzed the data the same way as earlier with the only difference that allele effects of the SNPs were tested

instead of the haplotype effects. All animals on the study were treated in accordance with guidelines established by the Iowa State University Committee on Animal Care.

Results

Mean values, their standard deviations, and 5% and 95% quantiles for milk production, milk fat percentage, concentrations of different fatty acids and fatty acid groups, and fatty acid indices are reported in table 3.3. There were 39 different milk fatty acids measured by GC. All fatty acid data were used to calculate the concentrations of individual fatty acids that were expressed as wt %, but the data reported are only for fatty acids present in milk at relatively high concentrations. The mean values for the major milk fatty acids in our study were similar to those reported by others (30, 52). The major fatty acid in milk was palmitic (16:0; 29.33 wt %) followed by oleic ($18:1^{c9}$; 23.55 wt %), stearic (18:0; 11.92 wt %), and myristic (14:0; 10.15 wt %) acids.

There were 7, 2, and 4 SNPs discovered in SLC27A6, FABP3, and FABP4, respectively (table 3.4). The discovered SNPs were validated by using the Sequenom MassARRAY system. The number of SNPs discovered was not large because of the small number of DNA samples (12 samples), used for sequencing. The majority of SNPs were intronic with a few synonymous and nonsynonymous mutations. After genotyping animals for discovered SNPs, there were 5, 3, and 3 haplotypes reconstructed for SLC27A6, FABP3, and FABP4, respectively, by using the PHASE program (table 3.5). The population frequencies of the reconstructed haplotypes were above 5 %.

F-tests were used to determine the overall haplotype effect of individual genes on milk fatty acid composition (table 3.6). Results with p-value ≤ 0.0167 were considered to

be significant, as determined by applying the Bonferroni adjustment to account for testing the association of genetic polymorphisms with milk fatty acid composition in each of three different genes. The overall haplotype effect of SLC27A6 was associated significantly with milk fat percentage, AI, concentrations of SFA, UFA, MUFA, SFA/UFA, concentrations of capric (10:0), lauric (12:0), myristic (14:0), and palmitic (16:0) acids. The overall haplotype effect of FABP3 was associated significantly with only pentadecylic (15:0) acid concentration and the elongation index (table 3.6). The FABP4 showed a significant association of its overall haplotype effect with the concentrations of SFA, UFA, MUFA, PUFA, SFA/UFA, the concentrations of linoleic ($18:2^{c9, c12}$) acid, CLA ($18:2^{c9, t11}$) and C18 desaturation index.

The pairwise comparisons between the haplotype effects of SLC27A6 on milk fat percentage and fatty acid composition (table 3.7) revealed that the haplotype h6 of SLC27A6 was the most desirable to select for animals producing healthier milk because the haplotype h6 of SLC27A6 was associated with 0.098 lower AI, 1.004 wt % lower SFA, 1.004 wt % higher UFA, 0.91 wt % higher MUFA concentrations, 0.072 lower SFA/UFA, 0.17 wt % lower capric (10:0) acid, 0.19 wt % lower lauric (12:0) acid, and 0.32 wt % lower myristic (14:0) acid concentrations compared with the effect of the haplotype h5 of SLC27A6. The effect of the haplotype h6 of SLC27A6 also was associated with 0.097 wt % lower capric (10:0) acid and 0.11 wt % lower lauric (12:0) acid concentrations compared with the effects of the haplotype h1 of SLC27A6. There was a tendency for the association of the haplotype h6 of SLC27A6 with 0.052 lower AI, 0.63 wt % lower SFA, 0.63 wt % higher UFA, 0.54 wt % higher MUFA concentrations, and 0.044 lower SFA/UFA compared with the effects of the haplotype h1 of SLC27A6.

The haplotype h9 of SLC27A6 was undesirable to select for animals producing milk with higher fat percentage because the haplotype h9 of SLC27A6 was associated with 0.17 and 0.15 % lower milk fat compared with the effects of the haplotypes h5 and h10 of SLC27A6 (table 3.7). On the other hand, the haplotype h9 of SLC27A6 was desirable to select for animals producing milk with lower concentration of palmitic (16:0) acid because the haplotype h9 of SLC27A6 was associated with 0.50 and 0.63 wt % lower palmitic (16:0) acid concentrations compared with the effects of the haplotypes h1 and h5 of SLC27A6.

Pairwise comparisons between the haplotype effects of FABP4 revealed that the haplotype h5 of FABP4 was the most desirable to select for animals producing milk with 0.57 and 0.51 wt % lower SFA, 0.57 and 0.51 wt % higher UFA, 0.48 and 0.40 wt % higher MUFA, 0.11 and 0.12 wt % higher PUFA concentrations, 0.04 and 0.037 lower SFA/UFA, 0.067 and 0.080 wt % higher linoleic ($18:2^{c9, c12}$) acid concentration, and 0.0051 and 0.0053 higher elongation index compared with the effects of the haplotypes h1 and h4 of FABP4 (table 3.7). The haplotype h5 of FABP4 also was associated with 0.035 wt % higher CLA ($18:2^{c9, t11}$) concentration compared with the effects of the haplotype h1 of FABP4. The pairwise comparisons between the haplotypes of FABP3 showed that the haplotype h1 of FABP3 was associated with 0.050 wt % higher concentration of pentadecylic (15:0) acid and 0.008 lower elongation index compared with the effect of the haplotype h2 of FABP3.

The allele substitution effect of DGAT1 A232K mutation was associated with milk fat percentage, AI, concentrations of SFA, UFA, MUFA, and PUFA, SFA/UFA, and concentrations of palmitic (16:0), stearic (18:0) and other fatty acids (table 3.8). The

significance was declared when $p \leq 0.05$. In particular, DGAT1 232K mutation was associated with 0.22 % higher milk fat, 0.10 higher AI, 1.05 wt % higher SFA, 1.05 wt % lower UFA, 0.88 wt % lower MUFA, 0.16 wt % lower PUFA concentrations, 0.086 higher SFA/UFA, 0.070 wt % higher lauric (12:0), 0.066 wt % higher myristoleic ($14:1^{c9}$), 0.87 wt % higher palmitic (16:0), 0.23 wt % lower stearic (18:0), 0.037 wt % lower oleic ($18:1^{c9}$), 0.038 wt % lower linoleic ($18:2^{c9, c12}$), 0.042 wt % lower CLA ($18:2^{c9, t11}$), 0.041 wt % higher caproic (6:0), 0.042 wt % higher caprylic (8:0), 0.086 wt % higher capric (10:0), 0.013 wt % higher tridecylic (13:0), 0.050 wt % higher pentadecylic (15:0) acid concentrations, 0.048 higher C14 desaturation index, and 0.015 lower elongation index compared with the effects of DGAT1 232A mutation. In addition, we tested FAS g. 17924G>A polymorphism for the association with milk fatty acid composition and the significance was declared when $p \leq 0.05$. Thus, the FAS g. 17924A allele was associated with 0.11 wt % higher myristic (14:0) and 0.17 wt % higher palmitic (16:0) acid concentrations.

Discussion

Fatty acid transport proteins, membrane-associated fatty acid binding proteins, and fatty acid translocase CD36 participate in fatty acid transport across a cell membrane (10, 11). The SLC27A6 corresponds to the group of fatty acid transport proteins. One study looked at polymorphisms in SLC27A1 known to be expressed in bovine mammary gland with respect to milk fat yield (39), but no significant associations were determined. The polymorphisms in fatty acid translocase CD36 that is known to be expressed in both murine and bovine mammary glands (7, 41), however, showed some significant

associations with fatty acid concentrations. Thus, a few genome-wide association studies in humans linked CD36 to some components of metabolic syndrome because of the ability of CD36 to affect fatty acid uptake in different tissues (1, 19, 36). It was shown that the polymorphisms in CD36 gene were associated with decreased plasma TAG and increased plasma HDL-C concentrations in African-Americans (34). In another human study, the plasma TAG lowering effect of fish oil was seen only in the individuals with certain polymorphisms in CD36 and the effect of those polymorphisms in the studied individuals was attributed to the increased uptake of fatty acids by extrahepatic tissues (35). Even though there is limited information about SLC27A6 polymorphisms and fatty acid uptake in different tissues, the fact that the polymorphisms in another fatty acid transporter CD36 were associated with fatty acid concentrations in human plasma lead us to believe in the potential role of SLC27A6 in fatty acid uptake in the mammary tissue.

The role of FABP1, SLC27A1, and CD36 in the fatty acid uptake into the mammary gland of lactating rats was studied using dietary CLA (23). It was determined that dietary CLA fed to rats decreased the concentrations of medium-chain SFA and palmitic (16:0) acid in milk. The change in milk fatty acid concentrations was accompanied by the decrease in the mRNA abundance of FABP1, SLC27A1, and CD36 in the mammary gland and by the increase in blood nonesterified fatty acid concentrations (23). The results of our study clearly indicate the importance of FABP1, SLC27A1, and CD36 in fatty acid uptake into mammary gland in rats and the fact that the uptake of medium-chain SFA and palmitic (16:0) acid was affected supports the results of our study that showed the association between the polymorphisms in SLC27A6 and the concentrations of capric (10:0), lauric (12:0), myristic (14:0), and palmitic (16:0)

acids in milk of dairy cows. The pairwise comparisons between the effects of the haplotypes of SLC27A6 revealed that the haplotype h6 always was associated with healthier fatty acid composition in milk compared with the effects of the haplotypes h1 and h5 of SLC27A6 (table 3.7). A "tag" SNP for the haplotype h6 of SLC27A6 was identified to be an intronic SNP385 located between exons 1 and 2. The allele C of SNP385 was present in the haplotype h6 of SLC27A6. In addition to being associated with unhealthy milk fatty acid composition, the haplotype h5 of SLC27A6 also was associated with 0.17 % higher milk fat compared with the effect of the haplotype h9 of SLC27A6. There were no significant associations between the haplotype h6 of SLC27A6 and milk fat percentage, meaning that selection for animals with haplotype h6 of SLC27A6 will not select for lower percentage of milk fat.

The FABP3 is one of the most abundant mRNA transcripts among FABPs in murine and bovine mammary glands (6, 7, 41). Moreover, the FABP3 expression was highly up-regulated during lactation in bovine mammary gland (6, 7). The deletion of FABP3 gene in mice, however, did not have any effect on mammary gland development or function (14), but the knockout mice had lower concentration of oleic ($18:1^{c9}$) acid in milk compared with the wild type mice. We were able to identify an association between the overall haplotype effect of SLC27A6 and the concentration of only pentadecylic (15:0) acid and the elongation index. Even though the size of the haplotype effect of SLC27A6 on the associated traits was not large, the fact that we had a significant association for the elongation index and a tendency for the association with oleic ($18:1^{c9}$) acid concentration ($p=0.018$, table 3.6) supports the correctness of our data.

Since the discovery of FABP4 in mice (27) and the association of FABP4 genetic polymorphisms with the plasma TAG concentrations in humans (53), there have been numerous studies that looked at polymorphism in FABP4 gene with regard to different lipid associated traits in farm animals. In particular, the polymorphism in FABP4 was associated with backfat and intramuscular fat thickness in beef cattle (3, 12), intramuscular fat thickness in pigs (22), and the amount of abdominal fat in chickens (54). The study that reported the effect of FABP4 genetic polymorphisms on fatty acid composition was in beef cattle with the association for palmitoleic ($16:1^{c9}$) acid concentration in adipose tissue (26). The only milk fatty acids that were associated with the FABP4 genetic polymorphisms in our study were linoleic ($18:2^{c9, c12}$) acid and CLA ($18:2^{c9, t11}$). The concentrations of SFA, UFA, MUFA, PUFA, SFA/UFA, and C18 desaturation index were associated with the FABP4 polymorphisms because of the association of linoleic ($18:2^{c9, c12}$) acid and CLA ($18:2^{c9, t11}$). The pairwise comparisons between the haplotype effects of FABP4 revealed that the haplotype h5 of FABP4 always was associated with healthier fatty acid composition of milk compared with the effects of the haplotypes h1 and h4 of FABP4. After comparing the haplotype sequences of FABP4, a "tag" SNP for the haplotype h5 of FABP4 was identified as a nonsynonymous SNP335 located in exon 3 that leads to the amino acid change from valine to methionine at residue 112 of FABP4 protein (tables 3.4 and 3.5). The A allele of SNP335 was present in the haplotype h5 of FABP4. In addition, three "tag" SNPs were identified for the haplotype h1 of FABP4 that was associated with unhealthy fatty acid composition in milk compared with the effects of the haplotype h5 of FABP4 (table 3.7). Those SNPs were SNP338 and SNP337 located in the intron between exons 3 and 4, and a synonymous SNP336 located

in exon 3. The C allele of SNP338, SNP337, and SNP336 was present in the haplotype h1 of FABP4.

A numerical comparison of the size of haplotype effects of SLC27A6, FABP3, and FABP4 and the size of allelic effects of DGAT1 A232K, and FAS g. 17924A>G on milk fat percentage and fatty acid composition was performed to determine which genetic polymorphisms had the largest effect (table 3.9). The size of the largest haplotype effect of SLC27A6 on milk fat percentage, AI, the concentrations of SFA and UFA, and SFA/UFA were numerically very similar to the size of the allelic effects of DGAT1 A232K mutation on those traits. Moreover, the size of the largest haplotype effects of LSC27A6 on the concentrations of capric (10:0) and lauric (12:0) acids were numerically much larger than the size of the allelic effects of DGAT1 A232K mutation on the same traits. At the same time, the size of the allelic effect of DGAT1 A232K on the concentration of palmitic (16:0) acid was numerically larger than the size of the haplotype effects of LSC27A6 on the concentration of the same fatty acid. The size of the haplotype effects of FABP4 on the concentrations of SFA, UFA, MUFA, and PUFA, SFA/UFA, and the concentration of linoleic ($18:2^{c9, c12}$) acid numerically was about one half of the size of allelic effects of DGAT1 A232K mutation on the same traits. Nevertheless, the genetic polymorphisms of FABP4 are still very valuable to select for animals producing milk with healthier fatty acid composition.

Conclusions

The results of our study show that the haplotype effects of SLC27A6 were associated significantly with milk fat percentage, AI, the concentrations of SFA, UFA, and MUFA,

SFA/UFA, and the concentrations of capric (10:0), lauric (12:0), myristic (14:0), and palmitic (16:0) acids. The haplotypes h5 and h6 of SLC27A6 that were the most desirable to select for animals producing milk with higher fat percentage and healthier fatty acid composition, respectively, did not have any significant associations with studied traits, indicating the possibility of selecting for the healthfulness of milk without affecting milk fat percentage.

The size of the haplotype effects of SLC27A6 on the studied traits was large and numerically similar to the size of allelic effects of DGAT1 A232K mutation that makes the polymorphisms in SLC27A6 as valuable as the of DGAT1 A232K mutation to select for animals producing milk with higher fat percentage and healthier fatty acid composition. The haplotype effects of FABP4 were associated significantly with the concentrations of SFA, UFA, MUFA, and PUFA, SFA/UFA, and the concentrations of linoleic ($18:2^{c9, c12}$) acid, CLA ($18:2^{c9, t11}$), and C18 desaturation index. A numerical comparison of the size of the haplotype effects of FABP4 with the size of the allelic effects of DGAT1 A232K mutation on milk fatty acid composition showed that the effects for FABP4 were not as large as those for DGAT1 A232K. The polymorphisms in FABP4, however, are still very valuable to select for milk healthfulness.

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Table 3.1 Gene information

Gene	BTA	Chromosomal position, cM	Gene length, bp	Transcript length, bp	Protein length, aa	Number of exons	Strand direction
SLC27A6	7	79996	24	2297	635	10	Reverse
FABP3	2	7648	101	724	133	4	Forward
FABP4	14	4390	42	631	134	4	Reverse

BTA, *Bos taurus* autosome; FABP3 and FABP4, fatty acid binding proteins-3 and -4; SLC27A6, solute carrier family 27, isoform A6

Table 3.2 Primer sequences and annealing temperatures for PCR

Gene	SNP	Forward primer	Reverse primer	PCR type[1]	Tm, °C
SLC27A6	**391**	GCATTTCTCATAGGGCTTGC	TACAGAGCCTCCCTCAGAGC	"Hot start"	60
SLC27A6	**390**	TCCTACCAGTGGATGTTACTGC	CCAAGTCTGTCTCGCTTTCC	Regular	55
SLC27A6	**385, 386, 387**	CCAATGTGAAAAGGAAGTTGG	GAAGTGGATCTGAGGCTTGG	Regular	55
SLC27A6	**383, 384**	CCAGCATTCTTTGGAGCAAC	ATACTCTACCTGCACCCACCAC	Regular	56
FABP3	**332**	GCTGACGTAGGCAAACTGG	CCCAACCTTGACCAGGAG	"Hot start"	58
FABP3	**334**	CAGCTCATGCTCATACCCTTC	GCTGGAGCTGGTGTAGACG	"Hot start"	60
FABP4	**335, 336, 337, 338**	AATTGCTAAGAACCTCAAAATAAGC	TATTGTCTCCTTCAATGTTGAGC	"Hot start"	60
DGAT1	**A232K**	TGGGCTCCGTGCTGGCCCTGATGGTCTA	TTGAGCTCGTAGCACAGGGTGGGGGCGA	"Hot start"	63

[1]"Hot start" PCR is a procedure that decreases non specific DNA amplification during the initial PCR cycles.
FABP3 and FABP4, fatty acid binding proteins-3 and -4; SLC27A6, solute carrier family 27, isoform A6

Table 3.3 Summary of milk fatty acid composition[1, 2]

Trait	Mean	Standard deviation	5% Quantile	95% Quantile
Milk production, kg/d	40.24	10.02	24.52	57.43
Milk fat, %	3.65	0.70	2.50	4.80
AI	2.07	0.46	1.27	2.80
SFA, wt %	63.93	4.47	55.57	70.29
UFA, wt %	36.07	4.47	29.71	44.43
MUFA, wt %	31.26	4.04	25.66	38.91
PUFA, wt %	4.81	0.92	3.46	6.42
SFA/UFA	1.81	0.34	1.25	2.37
4:0, wt %	2.47	0.76	1.29	3.80
6:0, wt %	1.55	0.35	0.90	2.05
8:0, wt %	1.00	0.23	0.57	1.35
10:0, wt %	2.47	0.63	1.35	3.47
12:0, wt %	2.94	0.73	1.61	4.10
13:0, wt %	0.18	0.08	0.07	0.33
14:0, wt %	10.15	1.60	6.80	12.26
14:1^{c9}, wt %	0.79	0.30	0.39	1.34
15:0, wt %	1.08	0.28	0.71	1.63
16:0, wt %	29.33	2.83	24.91	34.14
16:1^{c9}, wt %	1.75	0.41	1.16	2.47
17:0, wt %	0.63	0.09	0.50	0.77
18:0, wt %	11.92	2.37	8.44	16.14
18:2$^{c9, c12}$, wt %	3.24	0.70	2.23	4.47
18:1^{c9}, wt %	23.55	3.45	18.83	30.05
18:2^{c9,t11} (CLA), wt %	0.70	0.24	0.43	1.19
C14 Index[3]	0.07	0.02	0.04	0.11
C16 Index[4]	0.06	0.01	0.04	0.08
C18 Index[5]	0.66	0.05	0.59	0.74
Elongation index[6]	0.53	0.05	0.46	0.62

[1]Summary statistics were computed from 4397 milk samples collected from 551 cows.
[2]The data presented here are for only 16 fatty acids out of 39 fatty acids analyzed because the concentrations of the rest of fatty acids in milk were low.
[3]C14 Desaturation index = 14:1/ (14:0 + 14:1)
[4]C16 Desaturation index = 16:1/ (16:0 + 16:1)
[5]C18 Desaturation index = 18:1/ (18:0 + 18:1)
[6]Elongation index = (18:0 + 18:1)/ (16:0 + 16:1 + 18:0 + 18:1)

Table 3.4 SNPs used for the reconstruction of haplotypes in SLC27A6, FABP3, and FABP4 genes

SNP	Gene	Order in haplotype sequence	Nucleotide position, bp	Gene area	Alleles	Mutation type	A.A. Residue change
391	SLC27A6	1	70916	Intron 6-7	T/G	Intronic	None
390	SLC27A6	2	67847	Intron 6-7	T/C	Intronic	None
387	SLC27A6	3	16048	Intron 2-3	G/C	Intronic	None
386	SLC27A6	4	15975	Exon 2	T/C	Synonymous	None
385	SLC27A6	5	15740	Intron 1-2	A/C	Intronic	None
384	SLC27A6	6	390	Exon 1	C/T	Synonymous	None
383	SLC27A6	7	242	Exon 1	A/T	Nonsynonymous	K81M
332	FABP3	1	80	Exon 1	T/C	Synonymous	None
334	FABP3	2	3876	Intron 2-3	G/T	Intronic	None
338	FABP4	1	3767	Intron 3-4	T/C	Intronic	None
337	FABP4	2	3745	Intron 3-4	T/C	Intronic	None
336	FABP4	3	3711	Exon 3	G/C	Synonymous	None
335	FABP4	4	3691	Exon 3	G/A	Nonsynonymous	V112M

FABP3 and FABP4, fatty acid binding proteins-3 and -4; SLC27A6, solute carrier family 27, isoform A6

Table 3.5 Intragenic haplotypes and their frequencies

Gene	Haplotype	Sequence[1, 2]	Frequency[3]
SLC27A6	**h1**	$G_1C_2C_3C_4A_5C_6A_7$	0.44
SLC27A6	**h5**	$G_1C_2G_3T_4A_5C_6T_7$	0.14
SLC27A6	**h6**	$G_1C_2G_3T_4\underline{\mathbf{C}}_5C_6T_7$	0.11
SLC27A6	**h9**	$T_1T_2C_3C_4A_5C_6A_7$	0.07
SLC27A6	**h10**	$T_1T_2G_3T_4A_5C_6A_7$	0.18
FABP3	**h1**	T_1G_2	0.74
FABP3	**h2**	T_1T_2	0.07
FABP3	**h3**	C_1G_2	0.20
FABP4	**h1**	$\underline{\mathbf{C}}_1\underline{\mathbf{C}}_2\underline{\mathbf{C}}_3G_4$	0.50
FABP4	**h4**	$T_1T_2G_3G_4$	0.27
FABP4	**h5**	$T_1T_2G_3\underline{\mathbf{A}}_4$	0.24

[1]Subsripts indicate the order of SNPs in a particular haplotype for a particular gene
[2]The "tag" SNPs in the haplotypes of interest are highlighted in bold and underlined
[3]Only haplotypes with the population frequencies ≥ 5% were considered in the analysis
FABP3 and FABP4, fatty acid binding proteins-3 and -4; SLC27A6, solute carrier family 27, isoform A6

Table 3.6 Results of F-tests for the overall haplotype effect on milk fatty acid composition

Trait	SLC27A6		FABP3		FABP4	
	F-value	p-value	F-value	p-value	F-value	p-value
Milk fat	3.29	0.0059*	0.04	0.96	2.16	0.12
AI	3.38	0.005*	2.20	0.11	3.34	0.036
SFA	3.21	0.0071*	1.71	0.18	5.36	0.0048*
UFA	3.21	0.0071*	1.71	0.18	5.36	0.0048*
MUFA	2.96	0.012*	1.87	0.15	4.39	0.013*
PUFA	2.49	0.03	0.33	0.72	6.35	0.0018*
SFA/UFA	3.13	0.0082*	2.38	0.093	4.80	0.0084*
4:0	1.07	0.37	1.94	0.14	0.51	0.60
6:0	1.77	0.12	1.83	0.16	3.83	0.022
8:0	2.42	0.034	0.59	0.55	4.06	0.018
10:0	3.26	0.0063*	0.70	0.50	3.20	0.041
12:0	3.37	0.0051*	0.73	0.48	2.50	0.082
13:0	0.99	0.42	3.57	0.029	0.51	0.60
14:0	2.81	0.016*	0.70	0.50	1.71	0.18
$14:1^{c9}$	0.95	0.45	0.01	0.99	1.58	0.21
15:0	1.48	0.19	5.91	0.0028*	0.68	0.51
16:0	2.88	0.014*	2.96	0.052	0.42	0.66
$16:1^{c9}$	0.96	0.44	0.92	0.40	1.66	0.19
17:0	1.31	0.26	2.36	0.095	1.64	0.20
18:0	0.59	0.71	2.48	0.085	1.34	0.26
$18:1^{c9}$	1.77	0.12	4.03	0.018	1.31	0.27
$18:2^{c9, c12}$	2.31	0.042	1.13	0.32	5.20	0.0057*
$18:2^{c9, t11}$ (CLA)	0.83	0.53	0.52	0.60	4.91	0.0076*
Index 14	1.23	0.29	0.00	1.00	2.46	0.086
Index 16	1.3	0.26	0.67	0.51	2.98	0.051
Index 18	0.84	0.52	2.15	0.12	4.15	0.016*
Elongation index	1.81	0.11	5.09	0.0063*	0.13	0.88

*The Bonferroni adjustment was used to account for multiple testing on four different genes. The significance of haplotype effects was declared if $p \leq 0.0167$ (0.05 divided by the number of genes that was 3)
FABP3 and FABP4, fatty acid binding proteins-3 and -4; SLC27A6, solute carrier family 27, isoform A6
The numbers for the milk samples used in the analysis and the cows from which the samples were collected were the following: 4084 and 512; 4192 and 526; and 4133 and 518 for SLC27A6, FABP3, and FABP4, respectively.

Table 3.7 Pairwise comparisons of SLC27A6, FABP3, and FABP4 haplotype effects on milk fatty acid composition

SLC27A6								
H-H_b	**AI**	**SFA**	**UFA**	**MUFA**	**SFA/UFA**	**10:0**	**12:0**	**14:0**
H1-H6	0.052 (0.022) 0.017‡	0.63 (0.23) 0.0058‡	-0.63 (0.23) 0.0058‡	-0.54 (0.21) 0.01‡	0.044 (0.017) 0.01‡	0.097 (0.034) 0.0042	0.11 (0.038) 0.0048	NS
H5-H6	0.098 (0.026) 0.0002	1.004 (0.28) 0.0003	-1.004 (0.28) 0.0003	-0.91 (0.25) 0.0003	0.072 (0.021) 0.0006	0.17 (0.041) <.0001	0.19 (0.046) <.0001	0.32 (0.091) 0.0004

SLC27A6		
H-H_b	**Milk fat**	**16:0**
H1-H9	NS	0.50 (0.18) 0.0047
H5-H9	0.17 (0.051) 0.001	0.63 (0.19) 0.0009
H10-H9	0.15 (0.049) 0.0021	NS

FABP3		
H-H_b	**15:0**	**Elongation index**
H2-H1	-0.050 (0.016) 0.0027	0.008 (0.003) 0.0051

FABP4								
H-H_b	**SFA**	**UFA**	**MUFA**	**PUFA**	**SFA/UFA**	**18:2$^{c9, c12}$**	**18:2$^{c9, t11}$ (CLA)**	**C18 Index**
H1-H5	0.57 (0.18) 0.0018	-0.57 (0.18) 0.0018	-0.48 (0.16) 0.0039	-0.11 (0.034) 0.0022	0.04 (0.014) 0.0034	-0.067 (0.025) 0.0068	-0.035 (0.012) 0.0022	-0.0051 (0.002) 0.0088
H4-H5	0.51 (0.20) 0.0101	-0.51 (0.20) 0.0101	-0.40 (0.18) 0.029‡	-0.12 (0.038) 0.0012	0.037 (0.015) 0.013	-0.080 (0.027) 0.0029	NS	-0.0053 (0.0021) 0.013

[1]The first number out of three numbers in a cell is an estimate of the haplotype substitution effect expressed in wt % for all individual fatty acids and fatty acid groups excluding indices. The second number in the parenthesis is a standard error of an estimate of the haplotype substitution effect expressed in the same units as the estimate. The third number is a p-value.

*The Bonferroni adjustment for multiple comparisons was used, and significance was declared if p-value were ≤ 0.005, 0.0167, and 0.0167 for SLC27A6, FABP3, and FABP4, respectively (0.05 divided by the number of comparisons that were 10, 3, and 3 for SLC27A6, FABP3, and FABP4, respectively).

‡Tended to be significant (p-value ≤ 0.05).

H-H_b, indicates a pair of haplotypes for which the haplotype effects on milk fatty acid composition were compared with H_b, indicating the position of a haplotype used as a baseline.

FABP3 and FABP4, fatty acid binding proteins-3 and -4; NS, not significant; SLC27A6, solute carrier family 27, isoform A6

The numbers for the milk samples used in the analysis and the cows from which the samples were collected were the following: 4084 and 512; 4192 and 526; and 4133 and 518 for SLC27A6, FABP3, and FABP4, respectively.

Table 3.8 Allele substitution effects of DGAT1 A2232K and FAS g. 17924G>A polymorphisms on milk fatty acid composition

Trait	DGAT1 (A232K)			FAS (g. 17924G>A)		
	Estimate	Standard error	p-value	Estimate	Standard error	p-value
Milk fat, %	0.22	0.030	<.0001	0.0080	0.023	0.72
AI	0.10	0.019	<.0001	0.008	0.014	0.58
SFA, wt %	1.05	0.20	<.0001	-0.099	0.14	0.50
UFA, wt %	-1.05	0.20	<.0001	0.099	0.14	0.50
MUFA, wt %	-0.88	0.19	<.0001	0.11	0.13	0.41
PUFA, wt %	-0.16	0.037	<.0001	-0.011	0.028	0.70
SFA/ UFA	0.086	0.015	<.0001	-0.004	0.011	0.69
4:0, wt %	0.0046	0.025	0.86	-0.024	0.020	0.22
6:0, wt %	0.060	0.015	0.0001	-0.015	0.011	0.17
8:0, wt %	0.042	0.011	<.0001	-0.009	0.008	0.25
10:0, wt %	0.086	0.030	0.0042	-0.013	0.022	0.57
12:0, wt %	0.070	0.033	0.035	-0.003	0.024	0.90
13:0, wt %	0.013	0.003	<.0001	0.000	0.002	0.85
14:0, wt %	0.11	0.061	0.081	0.11	0.047	0.018
14:1^{c9}, wt %	0.042	0.015	0.0037	0.0012	0.0098	0.90
15:0, wt %	0.056	0.012	<.0001	-0.0042	0.0086	0.63
16:0, wt %	0.87	0.11	<.0001	-0.17	0.085	0.042
16:1^{c9}, wt %	0.087	0.022	<.0001	-0.0038	0.016	0.81
17:0, wt %	-0.0035	0.0032	0.27	0.0016	0.0023	0.49
18:0, wt %	-0.23	0.11	0.032	0.040	0.078	0.61
18:1^{c9}, wt %	-0.88	0.14	<.0001	0.036	0.10	0.73
18:2$^{c9, c12}$, wt %	-0.12	0.026	<.0001	-0.0077	0.020	0.70
18:2$^{c9, t11}$ (CLA), wt %	-0.028	0.013	0.028	0.0061	0.0093	0.51
C14 Index	0.0030	0.0013	0.016	-0.00022	0.00083	0.79
C16 Index	0.00099	0.00068	0.14	0.00024	0.00049	0.63
C18 Index	-0.004	0.002	0.062	0.000	0.002	0.83
Elongation index	-0.015	0.002	<.0001	0.002	0.001	0.23

DGAT1, diacylglycerol acyltransferase-1; FAS, fatty acid synthase

The numbers for the milk samples used in the analysis and the cows from which the samples were collected were the following: 2587 and 325; and 4003 and 500 for DGAT1 and FAS, respectively.

Table 3.9 Summary of the largest effects of polymorphisms in SLC27A6, FABP3, FABP4, and DGAT1 genes on milk fat percentage and milk fatty acid composition

Trait	Gene				
	SLC27A6	FABP3	FABP4	DGAT1	FAS
Milk fat, %	0.17	NS	NS	0.22	NS
AI	0.098	NS	NS	0.10	NS
SFA, wt %	1.004	NS	0.57	1.05	NS
UFA, wt %	1.004	NS	0.57	1.05	NS
MUFA, wt %	0.91	NS	0.48	0.88	NS
PUFA, wt %	NS	NS	0.12	0.16	NS
SFA/ UFA	0.072	NS	0.04	0.086	NS
4:0, wt %	NS	NS	NS	NS	NS
6:0, wt %	NS	NS	NS	0.060	NS
8:0, wt %	NS	NS	NS	0.042	NS
10:0, wt %	0.17	NS	NS	0.086	NS
12:0, wt %	0.19	NS	NS	0.070	NS
13:0, wt %	NS	NS	NS	0.013	NS
14:0, wt %	0.32	NS	NS	NS	0.11
$14:1^{c9}$, wt %	NS	NS	NS	0.042	NS
15:0, wt %	NS	0.050	NS	0.056	NS
16:0, wt %	0.63	NS	NS	0.87	0.17
$16:1^{c9}$, wt %	NS	NS	NS	0.087	NS
17:0, wt %	NS	NS	NS	NS	NS
18:0, wt %	NS	NS	NS	0.23	NS
$18:1^{c9}$, wt %	NS	NS	NS	0.88	NS
$18:2^{c9, c12}$, wt %	NS	NS	0.080	0.12	NS
$18:2^{c9, t11}$ (CLA), wt %	NS	NS	0.035	0.028	NS
C14 Index	NS	NS	NS	0.0030	NS
C16 Index	NS	NS	NS	NS	NS
C18 Index	NS	NS	0.0053	NS	NS
Elongation index	NS	0.008	NS	0.015	NS

FABP3 and FABP4, fatty acid binding proteins-3 and -4; FAS, fatty acid synthase; DGAT1, diacylglycerol acyltransferase-1; SLC27A6, solute carrier family 27, isoform A6

CHAPTER 4. EFFECTS OF GENETIC POLYMORPHISMS IN THE SREBP PATHWAY ON MILK FATTY ACID COMPOSITION IN DAIRY CATTLE

A paper to be submitted to Animal Genetics

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Abstract

Dietary saturated fatty acids (SFA) are known to increase plasma cholesterol concentrations in humans leading to the higher risk of developing cardiovascular diseases. In particular, individual SFA such as palmitic (16:0), myristic (14:0), and lauric (12:0) acids can raise plasma cholesterol concentrations much higher compared with the other SFA and their presence in milk at high concentrations is undesirable. The main objective of our study was to develop genetic markers that can be used to improve the healthfulness of bovine milk, that is known to contain high concentrations of SFA and palmitic (16:0) and myristic (14:0) acids. The sterol regulatory element binding protein-1c (SREBP-1c) is involved in the transcriptional regulation of lipogenesis, and its proteolytic activation is controlled by SREBP cleavage-activating protein (SCAP) and insulin-induced genes (Insig) that are all part of the SREBP pathway. We sequenced genes from the SREBP pathway to discover single nucleotide polymorphisms (SNPs) and reconstructed

haplotypes within each gene after genotyping cows for the discovered SNPs. The results of our study showed significant association of the overall haplotype effect of SREBP1 with the concentrations of myristic (14:0) and myristoleic (14:1^{c9}) acids, and C14 desaturation index. The overall haplotype effect of Insig1 was associated with the concentrations of polyunsaturated fatty acids (PUFA) and linoleic (18:2$^{c9, c12}$) acid. We did not detect any significant associations of SCAP genetic polymorphisms with milk fatty acid composition. In conclusion, the information about genetic polymorphisms in SREBP1 and Insig1 can be used to select animals producing milk with healthier fatty acid composition.

Introduction

Dietary saturated fatty acids (SFA) are known to raise plasma total cholesterol and low-density lipoprotein cholesterol concentrations in humans leading to the increased risk of developing cardiovascular diseases [1, 2]. Not all SFA, however, have the same effect on plasma cholesterol concentrations. Palmitic (16:0) and myristic (14:0) acids, for example, can raise plasma cholesterol concentrations much higher compared with the other SFA, and because of that their presence at high concentrations in human diets is undesirable [1]. The monounsaturated fatty acids (MUFA), however, are considered neutral with regard to plasma cholesterol concentrations, and their presence in human diets can be increased at the expense of SFA [1, 3, 4]. In recent years, a number of studies researched the possibilities of decreasing the concentration of atherogenic fatty acids in bovine milk by dietary and genetic means to improve the healthfulness of milk [5-10]. The genetic approach, however, is more plausible than the dietary approach because the conversion of

dietary unsaturated fatty acids (UFA) into milk UFA in ruminants is hampered by ruminal biohydrogenation [11]. The advantage of genetic improvement of milk healthfulness also is supported by the moderate to high heritabilities of milk fatty acids [7, 12-15]. To provide tools for the selection of animals with healthier milk fatty acid composition, we designed a study to discover genetic polymorphisms associated with milk fatty acid composition. The sterol regulatory element binding protein-1c (SREBP-1c) known to activate the expression of lipogenic genes such as acetyl-CoA carboxylase, fatty acid synthase, long chain fatty acid elongase, and stearoyl-CoA desaturase [16], and other genes from the SREBP pathway involved in the SREBP-1c activation were the focus of our study.

The SREBPs (SREBP-1a, SREBP-1c, and SREBP-2) are encoded by two different genes (SREBP1 and SREBP2), with SREBP-1a and SREBP-1c being produced from SREBP1 gene by alternative transcription [16]. The pathway of proteolytic activation of SREBPs was elucidated by studying SREBP-2 activation. It was established that in sterol-depleted cells SREBP cleavage activating protein (SCAP) transports SREBPs in the coat protein complex II (COPII)-coated vesicles from ER to Golgi, where N-terminal domain of SREBPs is released into the cytosol after a sequence of proteolytic cleavages catalyzed by site-1 and site-2 proteases [17, 18]. The cleaved SREBPs then enter the nucleus where they activate the transcription of genes from the fatty acid and cholesterol biosynthetic pathways. When ER cholesterol concentration rises above 5%, SCAP binds cholesterol and undergoes a conformational change that prevents SCAP-SREBP transport in COPII-coated vesicles to Golgi. As a result, N-terminal domain of SREBPs cannot be released and no transcriptional activation of sterol regulatory element

containing genes can occur. Another protein known as insulin-induced gene (Insig) was found to reside in ER membrane and to bind to SCAP in the presence of oxysterols leading to the inhibition of SCAP-SREBP transport to Golgi even in the sterol-depleted cells [17, 18]. There are two known genes (Insig-1 and Insig-2) that code for three different forms of Insig proteins (Insig-1, Insig-2a, and Insig-2b). In the presence of oxysterols, Insig-1 prevents SCAP-SREBP transport to Golgi. The Insig-1 also is transcriptionally activated by SREBPs, thus providing a feed-back mechanism by which nuclear SREBPs trigger buildup of their own inhibitor.

In contrast to SREBP-1a and SREBP-2, expression and nuclear abundance of SREBP-1c does not depend on cellular cholesterol concentration [19]. Moreover, overexpression of the nuclear form of SREBP-1c protein in the liver of transgenic mice caused TAG accumulation without any change in cholesterol concentration [20]. The activity of SREBP-1c is controlled by transcriptional regulation of its gene expression and by the proteolytic cleavage of SREBP-1c protein in Golgi. Transcriptional regulation of SREBP-1c is accomplished by liver X-activated receptors (LXRs), insulin, and glucagon [16]. The main role of LXRs is to activate oleate (18:1) production for the synthesis of cholesteryl esters. This increased oleate synthesis could be potentially achieved by up-regulating fatty acid elongase and the stearoyl-CoA desaturase activities as observed in transgenic mice overexpressing the nuclear form of SREBP-1a in their livers [21, 22]. LXRs are also a part of regulatory mechanism by which UFA can suppress fatty acid biosynthesis [16]. Insulin up-regulates SREBP-1c expression, leading to an increase in the rates of fatty acid biosynthesis as was demonstrated in rodents using cultures of isolated hepatocytes and adipocytes [23] whereas glucagon opposes the action

of insulin on lipogenesis by raising intracellular cAMP concentration [16]. It was shown that in liver insulin can increase SREBP-1c proteolytic activation by decreasing the mRNA abundance of Insig-2a protein that otherwise would be able to block SCAP-SREBP-1c transport from ER to Golgi and subsequently inhibit SREBP-1c activation [24]. Insulin did not change the transcription of Insig-1.

Mutations in the sterol-sensing domain of SCAP prevented sterol-induced binding of SCAP to Insig proteins and abolished feedback regulation of the SREBP processing by sterols in Chinese hamster ovary cell lines [25]. Considering this fact, the expression of genes of the SREBP pathway in bovine mammary gland [26], the importance of SREBP-1c activation for the induction of lipogenesis, and the necessity of interaction among SREBP-1c, SCAP, and Insig proteins for SREBP-1c proteolytic activation, we hypothesized that polymorphisms in SREBP-1, SCAP, and Insig-1 genes can be associated with differences in milk fatty acid composition in dairy cattle. The SREBP-1, Insig-1, and SCAP genes were sequenced to identify single nucleotide polymorphisms (SNPs). After genotyping animals for the discovered SNPs, the intragenic haplotypes were reconstructed and the associations between the haplotypes and milk fatty acid composition were tested for each gene separately. The results of the association tests are presented in this paper.

Materials and methods

Milk fatty acid analysis

Milk samples were collected during morning milking once a month throughout a 305-d lactation period and stored at -20 °C until further analysis. The extraction of milk fatty

acids with subsequent esterification into methyl esters for the analysis by gas chromatography (GC) was performed according to the procedure published by Chouinard et al. [27] with minor modifications. The procedure is based on the original milk lipid extraction method with hexane and isopropanol developed by Hara and Radin [28] and fatty acid methyl ester production method developed by Christie [29]. After thawing, milk samples were vortexed for 10 s and 25 mL aliquots were transferred to 50 mL teflon centrifuge tubes pre-rinsed with chloroform/ methanol (2/1 vol.vol). Following the centrifugation at 17,800 × *g* for 30 min at 4 °C, the liquid phase of the milk samples was removed and 18 mL of hexane/isopropanol (3/2 vol/vol) were added for every gram of lipids with subsequent vortexing for 1 min. To achieve a better separation of hexane phase containing lipids from water phase containing proteins, 12 mL of 6.7 % sodium sulfate (Na_2SO_4) solution were added for every gram of lipids and samples were vortexed for 1 min. After the centrifugation at 2,500 × *g* for 5 min at 4 °C, the hexane fraction containing lipids was removed into a scintillation vial pre-rinsed with chloroform/ methanol (2/1 vol/vol) and an aliquot containing 40 mg of lipids (400 μL) was transferred into an esterification vial. After the evaporation of hexane under nitrogen gas, 2 mL of hexane were added to the esterification vial followed by 40 μL of methyl acetate and vortexing for 30 s. The methylation step was initiated by the addition of 40 μL of sodium methoxide (0.4 mL of 5.4 M sodium methoxide in 1.75 mL of methanol) solution. The mixture was vortexed for 30 s and allowed to react for 10 min at room temperature. The reaction was terminated by the addition of 60 μL of oxalic acid (1 gram of oxalic acid/30 mL of diethyl ether) solution. The samples were centrifuged at 2,400 × *g* for 5 min at 4 °C and a clear hexane fraction that contained fatty acid methyl esters was placed into

GC vials, purged with nitrogen gas for 3 s, capped, and used for chromatographic analysis. In the above steps, capping of the vials that contained hexane lipid extract was preceded by purging with nitrogen gas for 3 s.

A Varian 3900 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA) equipped with CP-8400 auto-sampler, CP-8410 auto-injector, CP-1177 split/splitless injector, and flame ionization (FID) detector was used to analyze fatty acid methyl esters with helium as a carrier gas. One μL of fatty acid methyl esters in hexane was injected (split ratio 50:1) into a fused silica capillary column (Supelco^{TM}-2560 Capillary Column, 100 m x 0.25 mm i.d., with 0.2 μm film thickness) with the initial column temperature set at 70 °C and held for 4 min. Then, the column temperature was increased to 175 °C at the rate of 13 °C/min and held for 27 min with subsequent increase to 215 °C at the rate of 4 °C/min where it was held for 28 min [30]. The injector and detector temperatures were set constant at 220 °C. Peak area was measured by integration using Star Chromatography Workstation Version 6, and peaks were identified by comparing the retention times with separately run fatty acid methyl ester standards (Matreya LLC, Pleasant Gap, PA).

DNA extraction

Genomic DNA was extracted from 20 mL of blood obtained from the coccygeal vein of every dairy cow. The blood was collected into 10 mL EDTA Vacutainer tubes and kept at 4 °C. After transferring 20 mL of blood into 50 mL centrifuge tubes, the ice-cold 1X red blood cell (RBC) lysis buffer was added to fill in the tubes that were inverted and incubated on ice for 30 min. The samples were centrifuged at 300 × *g* for 5 min at 4 °C.

Supernatant was aspirated and 20 mL of ice-cold 1X RBC lysis buffer were added again followed by a light vortexing and 30 min and incubation on ice. After centrifugation and aspiration steps performed as mentioned earlier, 20 mL of room temperature PBS buffer were added to wash the pellet and the centrifugation step was repeated again. Supernatant was discarded and pellet was resuspended in PBS, transferred to cryovial, and store at -70 °C until further analysis. The DNA purification from the white blood cells was performed with DNeasy® Blood and Tissue Kit (Qiagen Inc., Valencia, CA) according to the instructions. The DNA concentration was measured using NanoDrop® ND1000 and dilutions were made with DNase free water to desired concentration.

SNP discovery and genotyping

The SREBP1, Insig1, and SCAP (table 4.1), were sequenced in exonic and some intronic regions to discover SNPs. A set of 12 DNA samples from cows that were daughters of 12 different sires was used for the SNP discovery. The PCR primers were designed with Primer3 (version 0.4.0) software [31]. Conventional or "Hot-Start" PCR was performed to amplify the DNA regions of interest. For the regular PCR, the reaction mixture contained 50 ng of genomic DNA, 13.6 μL of DNA grade water, 2 μL of DMSO, 1.5 μL of $MgCl_2$ (25 mM), 0.1 μL of forward and reverse primers (100 ng/μL), 0.5 μL of dNTP mix (40 mM), 5 μL of 5X buffer, and 0.2 μL of Tag DNA polymerase (5 U/μL) with the total volume of the reaction mixture equal to 25 μL. For the HotStarTag® PCR, the reaction mixture was almost the same as for the regular PCR with the only difference that 5 μL of 10X buffer was used instead of 5X buffer and 0.2 μL of HotStarTag DNA polymerase (5 U/μL) was used instead of Tag DNA polymerase. The PCRs were

performed in a DNA Engine thermal cycler (Bio-Rad) with the separate temperature cycle programs for the regular and HotStarTag® PCRs. The regular PCR was performed with the following program: 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, an optimal annealing temperature for a particular primer set (table 4.1) for 30 s, and 72 °C for 30 s with a final extension step at 72 °C for 10 min. The HotStarTag® PCR was performed with the following program: 95 °C for 15 min followed by 30 cycles of 94 °C for 1 min, an optimal annealing temperature for a particular primer set (table 4.2) for 1 min, and 72 °C for 1 min with a final extension step at 72 °C for 10 min. The optimal annealing temperature for a particular primer set was determined by a PCR temperature gradient. Before sequencing, the PCR product was cleaned up from unused primers and dNTPs with ExoSAP-IT® (USB Corporation, Cleveland, OH) that contained exonuclease 1 and shrimp alkaline phosphatase [32]. To identify SNPs, reverse and forward sequences representing 12 different samples were aligned using Vector NTI Advance™ 10. Genotyping of discovered SNPs was performed with Sequenom MassARRAY platform using 10 ng of genomic DNA dissolved in DNase-free water [33]. Haplotypes and their frequencies were estimated by using PHASE (version 2.1) program [34, 35]. Only samples with probability ≥0.9 for the best haplotype pair were used in the analysis.

Statistical analysis

A linear mixed model for longitudinal data was used to analyze the data with PROC MIXED procedure of SAS 9.1 (2002). The haplotype substitution model [36] used to test the association between intragenic haplotypes and milk fatty acid composition was the following:

$Y = \mu + dim + dim^2 + dim^3 + cg + dim*cg + dim^2*cg + dim^3*cg + \sum b_k H_k + sire + \varepsilon$

where y is the response variable; μ is the mean response at 0 dim for a cow with no copies of the haplotypes; dim, dim^2, and dim^3 are covariates describing the effects of days in milk on milk fatty acid composition; cg is a fixed effect of the contemporary group (cg=8 classes); H_k is a haplotype effect fitted as a covariate and coded as 0, 1, or 2 for 0, 1, or 2 copies of each haplotype present in an animal; b_k is the partial regression coefficient which corresponds to the haplotype substitution effect for the k^{th} haplotype as a deviation from the effect of the most frequent haplotype that is set to zero to have a full rank model; sire is a sire random effect; and ε is a residual error. The higher order terms for days in milk were introduced to account for a non-linear trend of the response variables over time. The cubic term of days in milk allows the response variable to have different slopes whenever the direction of its trend changes over time. When the higher order terms for days in milk were not significant, they were dropped from the model. Contemporary groups were created by combining herd (two herds) and season of calving (four seasons: December to February, March to May, June to August, and September to November) effects. The year of sampling was not included in the model to avoid redundancy because the season of calving is already containing some information about the year when a particular sample was collected. Haplotypes with a population frequency more than 0.05 were used in the analysis and those with frequency less than 0.05 were pooled in the "other" category. The CONTRAST statement in PROC MIXED was used to construct the F-tests of the overall haplotype effects and when the test was significant after applying a Bonferroni adjustment to account for multiple testing, pairwise comparisons between all haplotypes within each gene were performed by using

ESTIMATE statement and the estimates of the differences between haplotype effects, their standard errors, and p-values were reported. The REPEATED statement was used to indicate that milk samples were collected repeatedly for the same animal throughout the 305 d lactation period. Correlations between repeated measures taken on the same animal were modeled using a first order autoregressive covariance structure that assumes equal variances and correlations that decline exponentially with an increase in the distance between time points [37]. The covariance structure was selected based on the biology of the experiment and using Akaike's and Bayesian Information Criteria and residual log likelihood [38].

For the traits that were significantly associated with the overall haplotype effect, we performed pairwise comparisons between different haplotypes within the same gene to determine which haplotypes had significant effects on differences in milk fatty acid composition compared with the effects of other haplotypes. A Bonferroni adjustment was used to control the type I error rates for multiple comparisons, Bonferroni-adjusted significance levels were determined by dividing desired experiment-wide significance level (0.05) by the number of pairwise comparisons. The numbers of pairwise comparisons for SREBP1 and Insig1 genes were 3 and 3, respectively that resulted in Bonferroni-adjusted significance levels of 0.0167 each. We compared haplotype sequences within each gene based on their effects on milk fatty acid composition to determine "tag" SNPs that can be used as genetic markers. All animals on the study were treated in accordance with guidelines established by the Iowa State University Committee on Animal Care.

Results

There were 39 different milk fatty acids measured by GC and used to calculate the concentrations of individual fatty acids that were expressed as wt %. The data reported, however, are only for fatty acids present in milk at relatively high concentrations (table 4.3). Mean values, their standard deviations, and 5% and 95% quantiles for milk production, milk fat percentage, concentrations of different fatty acids and fatty acid groups, and fatty acid indices are reported. The mean values for the major milk fatty acids in our study were similar to those reported by others [6, 15], with palmitic (16:0; 29.33 wt %) acid being the major milk fatty acid followed by oleic ($18:1^{c9}$; 23.55 wt %), stearic (18:0; 11.92 wt %), and myristic (14:0; 10.15 wt %) acids.

The number of SNPs identified for SREBP1, Insig1, and SCAP was 5, 20, and 19, respectively (table 4.4). Those SNPs were validated using by Sequenom MassARRAY system. The number of SNPs for SREBP1 was not large because of the small number of animals (12 animals) used to discover SNPs. The majority of SNPs were intronic with two synonymous and one non-synonymous mutations. After genotyping animals for the discovered SNPs, the PHASE program was used to reconstruct intragenic haplotypes. There were 3, 3, and 2 haplotypes determined for SREBP-1, Insig-1, and SCAP genes, respectively, and their population frequencies were above 5 % (table 4.5).

F-tests were used to determine the overall haplotype effects on milk fatty acid composition for each gene separately (table 4.6). Results with p-value ≤ 0.0167 were considered to be significant, as determined by applying the Bonferroni adjustment to account for testing the association of genetic polymorphisms with milk fatty acid composition in each of three different genes. The overall haplotype effect of SREBP1

was associated significantly with the concentrations of myristic (14:0) and myristoleic ($14:1^{c9}$) acids and C14 desaturation index. The overall haplotype effect of Insig1 was associated significantly with the concentrations of MUFA, PUFA, and palmitic (16:0) and linoleic ($18:2^{c9, c12}$) acids. There was not any single trait that was associated significantly with the overall haplotype effect of SCAP.

The pairwise comparisons between the haplotype effects of SREBP1 revealed that the haplotype h1 of SREBP1 was the most desirable to select for animals producing healthier milk with lower concentration of myristic (14:0) acid, higher concentration of myristoleic ($14:1^{c9}$) acid, and higher C14 desaturation index. In particular, the haplotype h1 of SREBP1 was associated significantly with 0.23 wt % lower myristic (14:0) acid concentration compared with the effect of the haplotype h5 of SRABP1. Moreover, the haplotype h1 of SREBP1 was associated significantly with 0.036 wt % higher myristoleic ($14:1^{c9}$) acid concentration and 0.0033 higher C14 desaturation index compared with the effect of the haplotype h4 of SREBP1. At the same time, the haplotype h4 of SREBP1 was associated significantly with 0.043 wt % lower myristoleic ($14:1^{c9}$) acid concentration compared with the effect of the haplotype h5 of SREBP1.

The pairwise comparisons between the haplotypes of Insig1 revealed that the haplotype h23 of SREBP1 was the most desirable to select for animals producing healthier milk because it was associated significantly with 0.099 wt % higher PUFA and 0.083 wt % higher linoleic ($18:2^{c9, c12}$) acid concentrations compared with the effect of the haplotype h3 of Insig1. The pairwise comparisons between the haplotypes of Insig1 revealed no significant associations with MUFA and palmitic (16:0) acid concentrations because the significance of the overall haplotype effect for those traits was caused by the

haplotypes with the population frequencies less than 5 % that were not considered in pairwise comparisons. There were no pairwise comparisons performed between the haplotypes of SCAP because the overall haplotype effect of SCAP was not associated significantly with any of the traits.

Discussion

The fact that the transcriptional activity of stearoyl-CoA desaturase-1 (SCD-1) gene is regulated by the SREBP-1c transcription factor [16] sparked much interest into investigating the possible association between genetic polymorphisms in the SREBP1 and fatty acid composition in beef and milk. An 84 bp indel was discovered in intron 5 of the SREBP1 in Japanese Black cattle [39]. The indel presence resulted in the long and short forms of the SREBP1 being present in the cattle genome. The absence of the SREBP1 indel was associated with the higher percentage of MUFA and lower melting point in intramuscular adipose tissue of beef cattle [39]. The MUFA included only myristoleic (14:1), palmitoleic (16:1), and oleic (18:1) acids. The indel effect on fatty acid composition was explained by its possible role in micro-RNA production that could regulate the expression of some downstream genes involved in lipid metabolism. In a subsequent study, however, the effects of the indel present in SREBP1 gene on fatty acid composition of intramuscular adipose tissue in beef were not consistent with the earlier findings [40]. Moreover, the authors of the second study were able to determine the association of the 84 bp indel in the SREBP1 with lower concentrations of myristic (14:0) and palmitic (16:0) acids and higher C14 desaturation and elongation indices in only one group of animals. The association of the SREBP1 genetic polymorphism characterized by

the presence of the 84 bp indel in intron 5 also was studied in Italian Brown cattle with respect to variations in milk fatty acid composition, but no significant associations were found [41]. The chicken SREBP-1 genetic polymorphisms were tested for the association with the degree of fatness between lean and fat chicken lines, but no significant associations were found [42]. In a human study, polymorphisms in the SREBP-1 gene were associated with diabetes risk and plasma cholesterol concentrations [43]. In our study, the only fatty acids significantly associated with polymorphisms in the SREBP-1 gene were myristic (14:0) and myristoleic (14:1) acids and the C14 desaturation index. The pairwise comparisons between the SREBP1 haplotypes revealed two tag SNPs that allow distinguishing the most desirable haplotype h1 of SREBP1 from the rest of SREBP1 haplotypes. The tag SNPs were intronic SNP43 and nonsynonymous SNP46 with T alleles present in the h1 haplotype of SREBP1 for both SNPs. The nonsynonymous SNP43 was causing the amino acid change from leucine for T allele to proline for C allele at 852 residue of SREBP-1c protein. It is possible that the presence of leucine instead of proline at 852 residue of SREBP-1c protein leads to the increased ability of SREBP-1c transcription factor to activate the delta-9 desaturase transcription and as a result, leads to the increased concentration of myristoleic (14:1) acid, decreased concentration of myristic (14:0) acid, and increased C14 desaturation index. The delta-9 desaturases catalyze the desaturation of fatty acids with 12 to 19 carbon atoms [44]. The results of our study can be supported partially by what was reported for the association of the SREBP-1 genetic polymorphisms with beef fatty acid composition, but there are definitely many inconsistencies with respect to the SREBP1 genetic effect.

There were just a few studies that looked at polymorphisms in Insig2 gene with regard to obesity-related phenotypes and plasma cholesterol concentrations in humans [45, 46]. The significant association of Insig2 genetic polymorphisms was established only with plasma cholesterol concentrations [46]. There are no association studies that looked at polymorphisms in Insig1 gene to the best of our knowledge. In our study, the haplotype h23 of Insig1 was the most desirable to select for animals producing healthier milk because it was associated with the higher concentrations of PUFA and linoleic ($18:2^{c9, c12}$) acids. We were able to identify a group of "tag" SNPs for the h23 haplotype of Insig1 that included all SNPS from which the haplotype was reconstructed except for SNP81, SNP120, SNP121, and SNP133. All the "tag" SNPs for the haplotype h23 of Insig1 were intronic except for SNP96 that was a synonymous mutation located in exon 4. We also were able to identify a "tag" SNP for the haplotype h3 of Insig1 that was an intronic SNP133. The effects of Insig1 genetic polymorphisms on fatty acid composition can be attributed to the Insig1 altered maturation of SREBP-1c and consequently, changes in the expression of SREBP-1c target genes that lead to the observed phenotypes.

A study on a diet-induced milk fat depression in dairy cows highlighted a potential role of SREBP1 in the regulation of lipid biosynthesis in lactating mammary gland by showing a decrease in SREBP1, SCAP, Insig1, and Insig2 gene expressions after feeding a diet rich in vegetable oil [47]. Based on the results of this study, it is difficult to see, however, whether Insig1 or Insig2 played any role in the regulation of SREBP1 protein activation, because, if it was the case, the expression of either Insig1 or Insig2 or both isoforms should had been increased. It could be also possible that the low expression levels for Insig1 and Insig2 were seen as a result of decreased SCAP

expression. In another study, there was an up-regulation in the expression of SREBP1, SREBP2, SCAP, Insig1, and Insig2 during the lactation, but the information is still incomplete to make any conclusions about potential involvement of any of the above proteins from the SREBP pathway in the regulation of lipid biosynthesis in bovine mammary gland [26]. The importance of Insig-1 in the regulation of SREBP processing was demonstrated first in vitro with regard to cholesterol biosynthesis [48]. Later, the importance of both Ising1 and Insig2 proteins in the suppression of hepatic lipogenesis was demonstrated in vivo [49]. The information about functionality of the SREBP pathway in the mammary gland and its importance in the regulation of lipid biosynthesis is lacking. In mice, it was shown that SREBP-1a and SREBP-1c are expressed in the mammary tissue and their expression is increased during lactation [50]. In addition to earlier described regulatory mechanisms for lipid biosynthesis involving SREBP-1c that are assumed to be true in lactating mammary gland, some studies suggested a potential role for insulin in the regulation of lipid biosynthesis via PI3 kinase pathway with protein kinase B (Akt1) being a possible downstream effector. Overexpression of a constitutively active form of Akt1 in mice during lactation results in precocious lipid droplet formation in mammary epithelial cells with a significant increase in milk lipid concentration that reaches 60-70% on a wet weight bases as compared to only 25-30% in wild type mice [51]. Based on the recent discoveries of Akt role in the regulation of SREBP activation [52-54], it was proposed [50] that insulin-mediated up-regulation of lipid biosynthesis via Akt is achieved by either Akt stimulated proteolytic activation of SREBP-1c or Akt-stimulated decrease in the degradation of a nuclear form of SREBP-1c in the nucleus or both.

There were a few studies that looked at the associations of SCAP genetic polymorphisms with obesity- and atherosclerosis-related phenotypes in humans, but no significant associations were found [55-57]. In a study with mutant Chinese hamster ovary cell lines, a few mutations identified in SCAP gene were related to abolished interaction between SCAP and SREBP proteins and failed inhibition of cholesterol biosynthesis even in the presence of sterols in the culture media [25]. In our study, we were not able to find any significant associations between SCAP genetic polymorphisms and milk fatty acid composition. Moreover, we used 19 SNPs to reconstruct haplotypes within SCAP gene and identified only two haplotypes that differ by alleles of SNP52. Our results show that the genetic polymorphisms in SCAP are not abundant because of the importance of maintaining the integrity of SCAP protein for its function.

Conclusions

The results of our study showed that the haplotype h1 of SREBP1 was the most desirable to select for animals producing healthier milk because the haplotype h1 of SREBP1was associated significantly with lower concentration of myristic (14:0) acid, higher concentration of myristoleic ($14:1^{c9}$) acid, and larger C14 desaturation index. Two "tag" SNPs for the haplotype h1 of SREBP1 were identified, with one being a nonsynonymous mutation that might be responsible for the effects attributed to the haplotype h1 of SREBP1 on milk fatty acid composition. The haplotype h23 of Insig1 was the most desirable to select for milk healthfulness because it was associated significantly with higher concentrations of PUFA and linoleic ($18:2^{c9, c12}$) acid in milk. There were 15 "tag" SNPs identified for the haplotype h23 of Insig1. There were no significant associations

with milk fatty acid composition determined for SCAP. In conclusion, the information about the genetic polymorphisms in the SREBP pathway can be used to develop genetic markers to select for animals producing healthier milk.

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Table 4.1 Gene information

Gene	BTA	Chromosomal position, cM	Gene length, bp	Transcript length, bp	Protein length, aa	Number of exons	Strand direction
SREBP1	19	50	15922	3871	1146	19	Forward
Insig1	4	101	12710	2762	276	6	Forward
SCAP	22	64	27746	3880/ 3991	1244/ 1281	21/ 22	Reverse

BTA, *Bos taurus* autosome; Insig1, insulin-induced gene-1; SCAP, sterol regulatory element binding protein cleavage-activating protein; SREBP1, sterol regulatory element binding protein-1

Table 4.2 Primer sequences and annealing temperatures for PCR

Gene	SNP	Forward primer	Reverse primer	Tm, °C
SREBP1	**42, 43**	CAGGTGCTTTTGGTAGAGGAG	GCAGGACCTAAGGACAGGAG	58
SREBP1	**44**	GCTGGTGCTCTTCTCCTTG	AGAGACGTTGCAGCAGGTG	60
SREBP1	**46**	CATCACCATGCAGTGAGGAG	CATGCTGGAGCTGATGGAG	58
SREBP1	**47**	GCAGCTTCTCCATCAGCTC	CCCTTACCTTGTCAATGGAG	56
Insig1	**79**	GGTGGTGAACCAGAGCAGAG	GAGAAGATGGTGGCGATGAC	56
Insig1	**81, 85**	TGCACTTCCGTCTTCATTCTC	TGGAGTCAGCCAGGTACTGAG	56
Insig1	**93**	TCCAGAAGGCAGAGCAGAAG	GGATTCATTTCAACCACGAAAC	56
Insig1	**94, 95, 96, 99, 100**	AGTTGTTTCGTGGTTGAAATG	TGTGAATCAACAATAAGAAGTGC	56
Insig1	**103**	TACCCATGAAGCTGAGATGC	CAGGCAAACCAAGCTGAC	58
Insig1	**109, 110, 111, 112**	CTGGGTCAGCTTGGTTTGC	ATGTTGCACGCTCCTTCATC	60
Insig1	**113**	GGGTGGTTGCTGTCATAGG	CACCAGCCTCTTCCAGTTTC	58
Insig1	**120, 121**	TTTGGATGTTACTGTTGTGTCTTG	GGCTCCTCACTACTGCACG	56
Insig1	**127**	CCCTCCTTCAGTCTGTCCTG	TGCTCCTGCAACAACAGC	58
Insig1	**129**	CACCTTGCTAGACTCCCTTG	GCAGACAACAGACACGAACTC	56
Insig1	**132, 133**	AGCAGTACCACCTCCTGTCC	CAACTTGGCATTTAATAACACGAG	56
SCAP	**51, 52**	TAGTGTAAACCATACCCTCAATGTG	GGAGTGGTGAATTCCACAGG	58
SCAP	**54**	CCTTGCCAGGAACAGGAC	CTGCACGCTTTACCTGACTG	56
SCAP	**56**	TGCTGTTTTATCCTCACAGTGC	TTAGAGCAAAGCTGACATCAAGTTAC	56
SCAP	**57**	CTTCTCCACCCTCTCTGTGC	CACTCACAAACGCAGACTACC	56
SCAP	**58, 59**	GGAGTTGATGGTTCCTGCTC	ATGAAGCCAACCAGCAAAG	58
SCAP	**60**	CTGCATGGGATTACCTACCTG	CCTCTCCACTTGGCACTCTC	56
SCAP	**61**	TGACTTCCTTCCAGAGAGTGC	AGAATGACACCTGACCTCTGC	58
SCAP	**63, 64**	GCAGAGGTCAGGTGTCATTC	CACTTCACAAAGGAAGATGTGC	58
SCAP	**65**	AACGCTAGCACTTTCTCTAACAAAC	ATGGCTGATTCATGCTGATG	56
SCAP	**67, 68**	TTTTCTCCACATCCTCTCCAG	GATCAGTGACTCCAACACACG	58
SCAP	**72**	GGTGCTGGAGAATTCACATCC	CATCTGCTCCCACTCCTCAG	56
SCAP	**73, 75**	ACCTCCTGACATCGTCTGC	GGGAGTAATGGAAATCACAGG	23
SCAP	**393**	GTCCTTAGGGTTGGAGCATTTG	AGCTGGGAGGCTCAGTAATGG	60‡
SCAP	**395**	CTAACCACCTCCTCCTCAGC	CAGACTCCTCTCCCTGATGAC	56

Insig1, insulin-induced gene-1; SCAP, sterol regulatory element binding protein cleavage-activating protein; SREBP1, sterol regulatory element binding protein-1

Table 4.3 Summary of milk fatty acid composition[1, 2]

Trait	Mean	Standard deviation	5% Quantile	95% Quantile
Milk production, kg/d	40.24	10.02	24.52	57.43
Milk fat, %	3.65	0.70	2.50	4.80
AI	2.07	0.46	1.27	2.80
SFA, wt %	63.93	4.47	55.57	70.29
UFA, wt %	36.07	4.47	29.71	44.43
MUFA, wt %	31.26	4.04	25.66	38.91
PUFA, wt %	4.81	0.92	3.46	6.42
SFA/UFA	1.81	0.34	1.25	2.37
4:0, wt %	2.47	0.76	1.29	3.80
6:0, wt %	1.55	0.35	0.90	2.05
8:0, wt %	1.00	0.23	0.57	1.35
10:0, wt %	2.47	0.63	1.35	3.47
12:0, wt %	2.94	0.73	1.61	4.10
13:0, wt %	0.18	0.08	0.07	0.33
14:0, wt %	10.15	1.60	6.80	12.26
14:1^{c9}, wt %	0.79	0.30	0.39	1.34
15:0, wt %	1.08	0.28	0.71	1.63
16:0, wt %	29.33	2.83	24.91	34.14
16:1^{c9}, wt %	1.75	0.41	1.16	2.47
17:0, wt %	0.63	0.09	0.50	0.77
18:0, wt %	11.92	2.37	8.44	16.14
18:2$^{c9, c12}$, wt %	3.24	0.70	2.23	4.47
18:1^{c9}, wt %	23.55	3.45	18.83	30.05
18:2^{c9,t11} (CLA), wt %	0.70	0.24	0.43	1.19
C14 Index[3]	0.07	0.02	0.04	0.11
C16 Index[4]	0.06	0.01	0.04	0.08
C18 Index[5]	0.66	0.05	0.59	0.74
Elongation index[6]	0.53	0.05	0.46	0.62

[1]Summary statistics were computed from 4397 milk samples collected from 551 cows.
[2]The data presented here are for only 16 fatty acids out of 39 fatty acids analyzed because the concentrations of the rest of fatty acids in milk were low.
[3]C14 Desaturation index = 14:1/ (14:0 + 14:1)
[4]C16 Desaturation index = 16:1/ (16:0 + 16:1)
[5]C18 Desaturation index = 18:1/ (18:0 + 18:1)
[6]Elongation index = (18:0 + 18:1)/ (16:0 + 16:1 + 18:0 + 18:1)

Table 4.4 SNPs used for the reconstruction of haplotypes in SREBP1, Insig1, and SCAP genes

SNP	Gene	Order in haplotype sequence	Nucleotide position, bp	Gene area	Alleles	Mutation type	A.A. Residue change
43	SREBP1	1	8729	Intron 2-3	C/T	Intronic	None
42	SREBP1	2	8837	Intron 2-3	A/G	Intronic	None
44	SREBP1	3	11598	Intron 9-10	C/G	Intronic	None
46	SREBP1	4	13495	Exon 14	T/C	Nonsynonymous	L852P
47	SREBP1	5	13801	Intron 15-16	T/C	Intronic	None
79	Insig1	1	631	Intron 1-2	T/A	Intronic	None
85	Insig1	2	2681	Intron 2-3	A/C	Intronic	None
81	Insig1	3	2797	Intron 2-3	C/A	Intronic	None
93	Insig1	4	4280	Intron 3-4	G/A	Intronic	None
99	Insig1	5	4487	Intron 3-4	G/T	Intronic	None
96	Insig1	6	4683	Exon 4	C/T	Synonymous	None
95	Insig1	7	4772	Intron 4-5	C/G	Intronic	None
94	Insig1	8	4796	Intron 4-5	T/C	Intronic	None
103	Insig1	9	5157	Intron 4-5	C/T	Intronic	None
112	Insig1	10	5454	Intron 5-6	T/C	Intronic	None
111	Insig1	11	5501	Intron 5-6	G/A	Intronic	None
110	Insig1	12	5727	Intron 5-6	A/G	Intronic	None
109	Insig1	13	5790	Intron 5-6	A/G	Intronic	None
113	Insig1	14	6757	Intron 5-6	A/G	Intronic	None
120	Insig1	15	7413	Intron 5-6	G/A	Intronic	None
121	Insig1	16	7580	Intron 5-6	A/C	Intronic	None
127	Insig1	17	8900	Intron 5-6	A/G	Intronic	None
129	Insig1	18	9952	Intron 5-6	G/A	Intronic	None
133	Insig1	19	10782	Intron 5-6	T/C	Intronic	None
132	Insig1	20	10825	Intron 5-6	G/C	Intronic	None
52	SCAP	19	51	5' UTR/ Exon 1	T/G	Synonymous	None
51	SCAP	18	256	5' UTR/ Intron 1-2	T/C	Intronic	None
54	SCAP	17	747	Intron 2-3	C/G	Intronic	None
56	SCAP	16	1526	Intron 2-3	T/C	Intronic	None
57	SCAP	15	2043	Intron 2-3	A/T	Intronic	None
59	SCAP	14	2147	Intron 2-3	G/A	Intronic	None
58	SCAP	13	2357	Intron 2-3	A/G	Intronic	None
60	SCAP	12	2976	Intron 2-3	C/T	Intronic	None
61	SCAP	11	3634	Intron 2-3	A/C	Intronic	None
63	SCAP	10	4011	Intron 2-3	A/G	Intronic	None
64	SCAP	9	4020	Intron 2-3	G/A	Intronic	None
65	SCAP	8	5138	Intron 2-3	A/G	Intronic	None
67	SCAP	7	8259	Intron 2-3	T/C	Intronic	None

Insig1, insulin-induced gene-1; SCAP, sterol regulatory element binding protein cleavage-activating protein; SREBP1, sterol regulatory element binding protein-1

Table 4.4 (continued)

SNP	Gene	Order in haplotype sequence	Nucleotide position, bp	Gene area	Alleles	Mutation type	A.A. Residue change
68	SCAP	6	8296	Intron 2-3	G/A	Intronic	None
72	SCAP	5	11504	Intron 2-3	G/A	Intronic	None
73	SCAP	4	12615	Intron 2-3	A/G	Intronic	None
75	SCAP	3	12747	Intron 2-3	G/T	Intronic	None
393	SCAP	2	19727	Intron 9-10	G/A	Intronic	None
395	SCAP	1	21468	Intron 11-12	G/A	Intronic	None

Table 4.5 Intragenic haplotypes and their frequencies

Gene	Haplotype	Sequence[1, 2]	Frequency[3]
SREBP1	**h1**	$\underline{\mathbf{T}}_1G_2C_3\underline{\mathbf{T}}_4C_5$	0.61
SREBP1	**h4**	$C_1G_2G_3C_4C_5$	0.19
SREBP1	**h5**	$C_1\underline{\mathbf{A}}_2C_3C_4\underline{\mathbf{T}}_5$	0.20
Insig-1	**h3**	$A_1C_2C_3A_4T_5T_6G_7C_8T_9C_{10}A_{11}G_{12}G_{13}G_{14}G_{15}A_{16}G_{17}A_{18}\underline{\mathbf{C}}_{19}C_{20}$	0.35
Insig-1	**h7**	$A_1C_2A_3A_4T_5T_6G_7C_8T_9C_{10}A_{11}G_{12}G_{13}G_{14}A_{15}C_{16}G_{17}A_{18}T_{19}C_{20}$	0.28
Insig-1	**h23**	$\underline{\mathbf{T}}_1\underline{\mathbf{A}}_2C_3\underline{\mathbf{G}}_4\underline{\mathbf{G}}_5\underline{\mathbf{C}}_6\underline{\mathbf{C}}_7\underline{\mathbf{T}}_8\underline{\mathbf{C}}_9\underline{\mathbf{T}}_{10}\underline{\mathbf{G}}_{11}\underline{\mathbf{A}}_{12}\underline{\mathbf{A}}_{13}A_{14}G_{15}\underline{\mathbf{A}}_{16}\underline{\mathbf{A}}_{17}G_{18}T_{19}\underline{\mathbf{G}}_{20}$	0.33
SCAP	**h1**	$G_1G_2G_3A_4G_5G_6T_7A_8G_9A_{10}A_{11}C_{12}A_{13}G_{14}A_{15}T_{16}C_{17}T_{18}G_{19}$	0.11
SCAP	**h2**	$G_1G_2G_3A_4G_5G_6T_7A_8G_9A_{10}A_{11}C_{12}A_{13}G_{14}A_{15}T_{16}C_{17}T_{18}T_{19}$	0.75

[1]Subsripts indicate the order of SNPs in a particular haplotype for a particular gene
[2]The "tag" SNPs in the haplotypes of interest are highlighted in bold and underlined
[3]Only haplotypes with the population frequencies ≥ 5% were considered in the analysis
Insig1, insulin-induced gene-1; SCAP, sterol regulatory element binding protein cleavage-activating protein; SREBP1, sterol regulatory element binding protein-1

Table 4.6 Results of F-test for the overall haplotype effect on milk fatty acid composition

Trait	SREBP-1		Insig-1		SCAP	
	F-value	p-value	F-value	p-value	F-value	p-value
Milk fat	2.00	0.14	2.06	0.10	0.45	0.64
AI	2.36	0.09	2.25	2.25	0.49	0.61
SFA	0.83	0.44	2.95	0.032	1.00	0.37
UFA	0.83	0.44	2.95	0.032	1.00	0.37
MUFA	1.11	0.33	3.64	0.013*	0.90	0.41
PUFA	0.86	0.43	3.65	0.012*	0.53	0.59
SFA/UFA	0.74	0.48	2.73	0.043	0.81	0.44
4:0	0.45	0.64	0.33	0.81	1.57	0.21
6:0	0.59	0.56	0.58	0.63	0.74	0.48
8:0	0.94	0.39	1.18	0.32	0.42	0.66
10:0	2.61	0.07	1.05	0.37	0.58	0.56
12:0	2.93	0.05	1.17	0.32	0.84	0.40
13:0	1.19	0.30	1.69	0.17	0.31	0.73
14:0	7.87	0.0004*	0.44	0.73	0.15	0.86
$14:1^{c9}$	4.87	0.0079*	3.05	0.028	1.93	0.15
15:0	1.28	0.28	2.25	0.081	0.27	0.76
16:0	0.81	0.45	3.47	0.016*	1.24	0.29
$16:1^{c9}$	2.47	0.086	2.52	0.057	2.11	0.12
17:0	1.47	0.22	1.77	0.15	0.31	0.73
18:0	1.07	0.34	1.03	0.38	0.38	0.68
$18:1^{c9}$	1.89	0.15	1.42	0.24	0.34	0.71
$18:2^{c9, c12}$	2.06	0.13	4.45	0.0041*	0.12	0.89
$18:2^{c9, t11}$ (CLA)	0.48	0.62	2.35	0.072	1.70	0.18
Index 14	4.41	0.012*	2.66	0.047	2.31	0.10
Index 16	1.67	0.19	3.24	0.022	3.14	0.044
Index 18	0.20	0.82	1.04	0.38	1.22	0.30
Elongation index	0.90	0.41	1.92	0.13	0.13	0.88

*The Bonferroni adjustment was used to account for multiple testing on four different genes. The significance of haplotype effects was declared if $p \leq 0.0167$ (0.05 divided by the number of genes that was 3)

Insig1, insulin-induced gene-1; SCAP, sterol regulatory element binding protein cleavage-activating protein; SREBP1, sterol regulatory element binding protein-1

The numbers for the milk samples used in the analysis and the cows from which the samples were collected were the following: 3804 and 474; 3993 and 500; and 4074 and 511 for SREBP1, Insig1, and SCAP, respectively.

Table 4.7 Pairwise comparisons of SREBP1 and Insig1 haplotype effects on milk fatty acid composition

SREBP1				Insig1		
H-H_b	**14:0**	**14:1^{c9}**	**C14 Index**	**H-H_b**	**PUFA**	**18:2$^{c9, c12}$**
H5-H1	0.23 (0.057) <.0001	NS	NS	**H23-H3**	0.099 (0.032) 0.0024	0.083 (0.023) 0.0003
H1-H4	NS	0.036 (0.013) 0.0055	0.0033 (0.0011) 0.0031			
H5-H4	NS	0.043 (0.015) 0.0046	NS			

[1]The first number out of three numbers in a cell is an estimate of the haplotype substitution effect expressed in wt % for all individual fatty acids and fatty acid groups excluding indices. The second number in the parenthesis is a standard error of an estimate of the haplotype substitution effect expressed in the same units as the estimate. The third number is a p-value.

*The Bonferroni adjustment for multiple comparisons was used, and significance was declared if p-value was ≤ 0.0167 and 0.0167 for SREBP1 and Insig1, respectively (0.05 divided by the number of comparisons that were 3 and 3 for SREBP1 and Insig1, respectively).

H-H_b, indicates a pair of haplotypes for which the haplotype effects on milk fatty acid composition were compared with H_b, indicating the position of a haplotype used as a baseline.

Insig1, insulin-induced gene-1; SCAP, sterol regulatory element binding protein cleavage-activating protein; SREBP1, sterol regulatory element binding protein-1

The numbers for the milk samples used in the analysis and the cows from which the samples were collected were the following: 3804 and 474; and 3993 and 500 for SREBP1 and Insig1, respectively.

CHAPTER 5. GENERAL CONCLUSIONS

General Discussion

The main objective of my dissertation research project was to identify single nucleotide polymorphisms (SNPs) that are associated with bovine milk fatty acid composition. The information about those SNPs can be used by animal breeders to select for animals producing milk with a healthier fatty acid profile. In recent years, much attention has been devoted to controlling fatty acid composition of human diets because of the association of high concentrations of dietary saturated fatty acids (SFA) with the increased incidence of cardiovascular diseases (CVD), the number one cause of death worldwide. Bovine milk and other dairy products are one of the major sources of SFA in human diets. This fact underlies the importance of controlling fatty acid composition in milk and other dairy products to decrease the risk of developing CVD.

The number of studies that researched association between genetic polymorphisms and bovine milk fatty acid composition is limited [1-7]. One of the earlier studies that looked at the effects of genetics on milk fatty acid composition was conducted only for *Bos taurus* autosome 19 (BTA19) [6]. Another study used the whole-genome approach to find QTL for milk fatty acid composition [8, 9]. In our study, we used a candidate gene approach to find SNPs significantly associated with milk fatty acid composition. The glycerol-3-phosphate acyltransferases-1 and -4 (GPAT1 and GPAT4), 1-acylglycerol-3-phosphate acyltransferase-1 (AGPAT1), and phosphatidate phosphatase (LPIN1) genes from the triacylglycerol biosynthetic pathway were studied to test association of the polymorphisms in those genes with milk fatty acid composition. The

overall haplotype effect of GPAT4 was significantly associated with the concentrations of capric (10:0), lauric (12:0), palmitic (16:0), and oleic ($18:1^{c9}$) acids and as a consequence with the concentrations of SFA, unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), SFA/UFA, C16 and C18 desaturation indices, and the atherogenic index (AI). The overall haplotype effect of GPAT1 was significantly associated with milk fat percentage, the concentrations of caproic (6:0), caprylic (8:0), capric (10:0), tridecylic (13:0), margaric (17:0), and myristoleic ($14:1^{c9}$) acids and C14 desaturation index. The overall haplotype effect of AGPAT1 showed significant associations with the concentrations of PUFA, caproic (6:0), margaric (17:0), and linoleic ($18:2^{c9, c12}$) acids, conjugated linoleic acid (CLA, $18:2^{c9, t11}$), and myristoleic ($14:1^{c9}$) acid. The overall haplotype effects of LPIN1 were associated only with myristoleic ($14:1^{c9}$) acid concentration and C14 desaturation index.

After numerical comparison of the size of haplotype effects of GPAT4 and GPAT1 and the size of allelic effects of DGAT1 A232K on milk fat percentage and fatty acid composition, we determined the haplotype effect of GPAT4 was associated with at least the same or larger differences in AI (0.13), concentrations of SFA (1.48 wt %), UFA (1.48 wt %), MUFA (1.34 wt %), PUFA (0.23 wt %), SFA/UFA (0.12), and lauric (12:0; 0.23 wt %) acid concentration compared with the effects of DGAT1 A232K mutation. The haplotype effect of GPAT4 also was associated with at least the same or larger difference in capric (10:0; 0.21 wt %) acid concentration compared with the GPAT1 haplotype effects.

The solute carrier family 27 (SLC27), isoform A6 and fatty acid binding proteins-3 and -4 (FABP3 and FABP4) involved in fatty acid uptake into mammary gland and

fatty acid transport inside the mammary epithelial cells were studied to test the association of the polymorphisms in those genes with milk fatty acid composition. The results of our study showed that the overall haplotype effect of SLC27A6 was associated significantly with the percentage of milk fat, the concentrations of SFA, UFA, MUFA, SFA/UFA, the concentrations of capric (10:0), lauric (12:0), myristic (14:0), and palmitic (16:0) acids in milk. The overall haplotype effect of FABP4 was associated significantly with the concentrations of SFA, UFA, MUFA, PUFA, SFA/UFA, the concentrations of linoleic ($18:2^{c9, c12}$) acid, conjugated linoleic ($18:2^{c9, t11}$) acid, and C18 desaturation index. The overall haplotype effect of FABP3 was associated significantly with the concentrations of only pentadecylic (15:0) acid and elongation index.

Numerical comparison of the size of the haplotype effects of SLC27A6 and the size of allelic effects of DGAT1 A232K on milk fat percentage and fatty acid composition, we determined that the size of the largest haplotype effect of SLC27A6 on milk fat percentage, AI, the concentrations of SFA and UFA, and SFA/UFA were numerically very similar to the size of the allelic effects of DGAT1 A232K mutation on those traits. Moreover, the size of the largest haplotype effects of LSC27A6 on the concentrations of capric (10:0) and lauric (12:0) acids were numerically much larger than the size of the allelic effects of DGAT1 A232K mutation on the same traits.

The sterol regulatory element binding protein-1c (SREBP-1c) is involved in the transcriptional regulation of lipogenesis, and its proteolytic activation is controlled by SREBP cleavage-activating protein (SCAP) and insulin-induced genes (Insig) that are all part of the SREBP pathway. The results of our study showed the significant association of the overall haplotype effect of SREBP1 with the concentrations of myristic (14:0) and

myristoleic ($14:1^{c9}$) acids, and C14 desaturation index. The overall haplotype effect of Insig1 was associated with the concentrations of PUFA and linoleic ($18:2^{c9, c12}$) acid. We did not detect any significant associations of SCAP genetic polymorphisms with milk fatty acid composition.

In conclusion, we were able to identify genetic polymorphisms in GPAT4, GPAT1, and SLC27A6 genes that were associated with the differences in milk fatty acid composition and the size of those differences for certain fatty acids was very large when numerically compared with the effects of DGAT1 A232K mutation. The associations of the polymorphisms for the other genes were valuable as well. The results of this study provide the information about genetic polymorphisms that can be used to develop genetic markers for the selection of animals producing milk with the healthier fatty acid profile.

Future Research

In our study, we used a candidate gene approach to discover genetic polymorphisms that are associated significantly with bovine milk fatty acid composition. Even though it is a good approach for the discovery of the associations of interest [10], the whole-genome studies could provide a better view of the whole picture. One of the first studies that searched for the QTLs associated with milk fatty acid composition focused only on BTA19 [6]. At that time, the QTL located in the same region of the chromosome as fatty acid synthase gene was identified. Later, the first whole-genome study that used SNPs as genetic markers was conducted to search for QTLs associated with bovine milk fatty acid composition [8, 9]. One of the main disadvantages of both studies was a small number of DNA markers used to cover each chromosome. In the future, it is desirable to do studies

with 50K or 100K SNP chips to genotype each animal for those numbers of SNPs and improve the accuracy of detecting and positioning QTLs for milk fatty acid composition. Ideally, studies that use chips with more than a million SNPs would be desirable to get better results.

As for the candidate gene approach, it is necessary to use a DNA sample set from more than 12 animals to increase chances of discovering more SNPs. Then, choosing between 10 and 20 equally spaced SNPs throughout a gene and genotyping animals for those SNPs with subsequent haplotype reconstruction within each gene and association analysis is a good approach to evaluate the effects of genetic polymorphisms for a particular gene on milk fatty acid composition. In the event of significant associations, further gene sequencing to detect nonsynonymous and possibly causative SNPs should be continued.

It is known that a quantitative trait such as milk fatty acid composition is determined by a large number of genes and by the interactions between those genes. So, it is important to test the associations between the phenotype and SNP interactions in the whole-genome studies or haplotype interactions within certain biosynthetic pathways when a candidate gene approach is used. Inexpensive and efficient ways to genotype animals for the SNPs of interest is an issue when the candidate gene approach is used. Even though the Sequenom MassARRAY system is a very efficient way to do genotyping, the fact that amplifying DNA in the areas of the genome rich in GC base pairs could reduce the PCR yield and consequently decrease the quality of genotypic data that would encourage researchers to look for the alternative ways to genotype their animals.

The phenotypic data set should be larger. In our study, number of daughters per sire was relatively small that did not allow good estimation of sire effects. In the future, the number of animals from which the whole-lactation monthly milk samples are collected should be at least doubled to 1,000. Another issue that comes with the increased number of milk samples to be analyzed for fatty acid composition is the high cost of using gas chromatography for this type of analysis. The alternative approach would be to use infrared spectroscopy after developing the prediction equations based on the milk fatty acid data obtained from GC or to use high performance liquid chromatography and mass spectrometry to obtain much broader information about composition of milk samples.

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APPENDIX A. SUPPLIMENTARY INFORMATION FOR CHAPTER 2

Table A1 Pairwise comparisons of GPAT4 haplotype effects on milk fatty acid composition

Trait	Haplotype	h1	h2	h3	h6	h8	h10
AI	h1		0.024 0.031	-0.024 0.021	0.047 0.025	-0.085 0.033	-0.025 0.024
	h2	0.4509		-0.047 0.029	0.023 0.034	-0.109 0.041	-0.049 0.032
	h3	0.2487	0.1079		0.070 0.024	-0.062 0.033	-0.002 0.020
	h6	0.0655	0.4995	0.0035		-0.132* 0.036	-0.072 0.026
	h8	0.0101	0.0078	0.0605	0.0002*		0.060 0.034
	h10	0.2909	0.1286	0.9363	0.005	0.0803	

Trait	Haplotype	h1	h2	h3	h6	h8	H10
SFA	h1		0.095 0.331	-0.262 0.216	0.640 0.267	-0.842 0.347	-0.255 0.253
	h2	0.7729		-0.357 0.309	0.545 0.358	-0.937 0.430	-0.350 0.339
	h3	0.2269	0.2487		0.902* 0.253	-0.580 0.345	0.007 0.215
	h6	0.0166	0.1285	0.0004*		-1.482* 0.375	-0.895* 0.269
	h8	0.0156	0.0295	0.0926	<.0001*		0.587 0.360
	h10	0.3137	0.3026	0.9736	0.0009*	0.1034	

*The Bonferroni adjustment for multiple comparisons was used, and significance was declared if p-value was ≤ 0.0033

The p-values for pairwise comparisons are below diagonal, and estimates with standard errors are above diagonal.

Table A1 (continued)

Trait	Haplotype	h1	h2	h3	h6	h8	h10
UFA	h1		-0.095 0.331	0.262 0.216	-0.640 0.267	0.842 0.347	0.255 0.253
	h2	0.7729		0.357 0.309	-0.545 0.358	0.937 0.430	0.350 0.339
	h3	0.2269	0.2487		-0.902* 0.253	0.580 0.345	-0.007 0.215
	h6	0.0166	0.1285	0.0004*		1.482* 0.375	0.895* 0.269
	h8	0.0156	0.0295	0.0926	<.0001*		-0.587 0.360
	h10	0.3137	0.3026	0.9736	0.0009*	0.1034	

Trait	Haplotype	h1	h2	h3	h6	h8	h10
MUFA	h1		-0.270 0.301	0.116 0.196	-0.741* 0.243	0.597 0.316	0.116 0.229
	h2	0.37		0.387 0.282	-0.471 0.326	0.867 0.391	0.386 0.309
	h3	0.5535	0.1708		-0.858* 0.231	0.481 0.314	0.000 0.195
	h6	0.0023*	0.149	0.0002*		1.339* 0.341	0.858* 0.245
	h8	0.0595	0.027	0.1256	<.0001*		-0.481 0.328
	h10	0.6123	0.2111	0.9993	0.0005*	0.1427	

Trait	Haplotype	h1	h2	h3	h6	h8	h10
PUFA	h1		0.176 0.060	0.148” 0.039	0.101 0.048	0.227* 0.063	0.147* 0.046
	h2	0.0034		-0.028 0.056	-0.075 0.065	0.051 0.078	-0.029 0.062
	h3	0.0002*	0.614		-0.047 0.046	0.079 0.062	-0.001 0.039
	h6	0.0368	0.2471	0.309		0.126 0.068	0.046 0.049
	h8	0.0003*	0.517	0.2075	0.065		-0.080 0.065
	h10	0.0014*	0.6354	0.9807	0.3486	0.2224	

Table A1 (continued)

Trait	Haplotype	h1	H2	h3	h6	h8	h10
SFA/UFA	h1		0.006 0.025	-0.022 0.016	0.047 0.020	-0.067 0.026	-0.014 0.019
	h2	0.8226		-0.028 0.023	0.042 0.027	-0.073 0.032	-0.019 0.026
	h3	0.178	0.2367		0.069* 0.019	-0.045 0.026	0.008 0.016
	h6	0.0188	0.1224	0.0003*		-0.115* 0.028	-0.061* 0.020
	h8	0.0101	0.0244	0.0804	<.0001*		0.054 0.027
	h10	0.4681	0.448	0.6137	0.0027*	0.0485	

Trait	Haplotype	h1	h2	h3	h6	h8	h10
8:0	h1		0.017 0.018	0.011 0.012	0.029 0.014	-0.026 0.019	-0.005 0.014
	h2	0.3447		-0.006 0.017	0.012 0.019	-0.043 0.023	-0.022 0.018
	h3	0.357	0.7108		0.018 0.014	-0.037 0.019	-0.016 0.012
	h6	0.0432	0.5278	0.1772		-0.056 0.020	-0.034 0.015
	h8	0.1612	0.0629	0.0468	0.0063		0.021 0.019
	h10	0.7062	0.2291	0.1704	0.0185	0.2745	

Trait	Haplotype	h1	h2	h3	h6	h8	h10
10:0	h1		0.126 0.048	0.051 0.031	0.069 0.039	-0.083 0.050	-0.012 0.037
	h2	0.009		-0.075 0.045	-0.057 0.052	-0.209* 0.062	-0.138 0.049
	h3	0.1043	0.0967		0.018 0.037	-0.134 0.050	-0.064 0.031
	h6	0.0755	0.2742	0.6279		-0.152 0.055	-0.081 0.039
	h8	0.1	0.0009*	0.0075	0.0054		0.071 0.052
	h10	0.7348	0.0052	0.0419	0.0379	0.1771	

Table A1 (continued)

Trait	Haplotype	h1	h2	h3	h6	h8	h10
12:0	h1		0.159 0.054	0.049 0.035	0.060 0.044	-0.068 0.057	-0.016 0.041
	h2	0.0034		-0.110 0.051	-0.099 0.059	-0.227* 0.070	-0.175* 0.055
	h3	0.1683	0.0295		0.011 0.041	-0.117 0.056	-0.064 0.035
	h6	0.1681	0.0917	0.7818		-0.129 0.061	-0.076 0.044
	h8	0.229	0.0013*	0.0379	0.0363		0.053 0.059
	h10	0.7039	0.0017*	0.0666	0.0853	0.3706	

Trait	Haplotype	h1	h2	h3	h6	h8	h10
14:0	h1		0.129 0.107	-0.018 0.069	0.019 0.086	-0.175 0.113	-0.170 0.081
	h2	0.2286		-0.147 0.101	-0.110 0.116	-0.305 0.139	-0.299 0.110
	h3	0.8005	0.1451		0.037 0.082	-0.158 0.112	-0.152 0.069
	h6	0.8232	0.345	0.6535		-0.195 0.122	-0.189 0.087
	h8	0.1209	0.0292	0.1577	0.1098		0.005 0.117
	h10	0.0362	0.0065	0.0271	0.0299	0.9633	

Trait	Haplotype	h1	h2	h3	h6	h8	h10
16:0	h1		-0.042 0.195	-0.312 0.128	0.228 0.157	-0.428 0.204	0.027 0.149
	h2	0.8288		-0.270 0.182	0.270 0.211	-0.386 0.253	0.069 0.200
	h3	0.0149	0.1383		0.540* 0.149	-0.116 0.203	0.339 0.127
	h6	0.1466	0.1997	0.0003*		-0.656* 0.221	-0.201 0.159
	h8	0.0367	0.128	0.5689	0.0031*		0.455 0.212
	h10	0.8564	0.7298	0.0078	0.2059	0.0324	

Table A1 (continued)

Trait	Haplotype	h1	h2	h3	h6	h8	h10
18:1^{c9}	**h1**		-0.27 0.24	0.01 0.15	-0.61* 0.19	0.19 0.25	-0.09 0.18
	h2	0.2583		0.28 0.22	-0.35 0.26	0.46 0.31	0.18 0.24
	h3	0.9263	0.2039		-0.63* 0.18	0.18 0.25	-0.11 0.15
	h6	0.0014*	0.179	0.0006*		0.80* 0.27	0.52 0.19
	h8	0.4477	0.138	0.4781	0.0029*		-0.28 0.26
	h10	0.6064	0.4698	0.4843	0.0069	0.2745	

Traits	Haplotype	h1	h2	h3	h6	h8	h10
CLA	**h1**		0.03 0.02	0.03 0.01	-0.01 0.02	0.05 0.02	0.03 0.02
	h2	0.1105		0.001 0.02	-0.04 0.02	0.02 0.03	-0.01 0.02
	h3	0.0105	0.9442		-0.04 0.02	0.02 0.02	-0.01 0.01
	h6	0.6096	0.0641	0.0068		0.06 0.02	0.03 0.02
	h8	0.0134	0.4397	0.3668	0.0079		-0.03 0.02
	h10	0.097	0.7391	0.5274	0.0399	0.2159	

Trait	Haplotype	h1	h2	h3	h6	h8	h10
C16 Index	**h1**		-0.0009 0.0011	0.0007 0.0007	-0.0019 0.0009	0.0008 0.0011	0.0013 0.0008
	h2	0.4261		0.0016 0.0010	-0.0010 0.0012	0.0017 0.0014	0.0022 0.0011
	h3	0.3353	0.1272		-0.0026* 0.0008	0.0001 0.0011	0.0006 0.0007
	h6	0.0299	0.3763	0.0019*		0.0027 0.0012	0.0032* 0.0009
	h8	0.4708	0.2323	0.9044	0.027		0.0005 0.0012
	h10	0.1077	0.0487	0.357	0.0003*	0.6632	

Table A1 (continued)

Trait	Haplotype	h1	h2	h3	h6	h8	h10
C18 Index	**h1**		0.004 0.004	0.001 0.002	-0.009* 0.003	0.002 0.004	0.001 0.003
	h2	0.2354		-0.003 0.003	-0.013* 0.004	-0.003 0.005	-0.004 0.004
	h3	0.6871	0.324		-0.010* 0.003	0.001 0.004	0.000 0.002
	h6	0.0022*	0.0007*	0.0004*		0.010 0.004	0.010* 0.003
	h8	0.6849	0.5588	0.8762	0.0106		-0.001 0.004
	h10	0.8208	0.3235	0.889	0.0012*	0.8163	

Table A2 Pairwise comparisons of GPAT1 haplotype effects on milk fatty acid composition

Trait	Haplotypes	h1	h2	h7	h8	h9	h12
Milk fat	**h1**		-0.108 0.057	-0.065 0.049	0.014 0.050	0.040 0.045	-0.061 0.048
	h2	0.0591		0.043 0.052	0.122 0.054	0.148* 0.047	0.047 0.053
	h7	0.1824	0.4103		0.079 0.039	0.105* 0.033	0.004 0.040
	h8	0.7793	0.023	0.0414		0.026 0.037	-0.075 0.044
	h9	0.3794	0.0019*	0.0015*	0.4852		-0.100 0.037
	h12	0.2053	0.3745	0.9119	0.0922	0.0064	

Trait	Haplotypes	h1	h2	h7	h8	h9	h12
6:0	**h1**		-0.046 0.027	-0.013 0.023	0.040 0.024	0.011 0.021	0.022 0.023
	h2	0.0881		0.033 0.024	0.086* 0.025	0.057 0.022	0.068 0.025
	h7	0.5571	0.1842		0.053 0.018	0.025 0.016	0.036 0.019
	h8	0.0944	0.0007*	0.0038		-0.028 0.017	-0.017 0.021
	h9	0.5911	0.0101	0.1089	0.109		0.011 0.017
	h12	0.3253	0.0068	0.0589	0.4073	0.5369	

Trait	Haplotypes	h1	h2	h7	h8	h9	h12
8:0	**h1**		-0.048 0.019	-0.020 0.016	0.009 0.017	0.002 0.015	0.008 0.016
	h2	0.0114		0.029 0.017	0.057* 0.018	0.051* 0.016	0.056* 0.018
	h7	0.2267	0.0997		0.029 0.013	0.022 0.011	0.028 0.013
	h8	0.5999	0.0014*	0.028		-0.006 0.012	-0.001 0.015
	h9	0.8789	0.0014*	0.0465	0.601		0.006 0.012
	h12	0.6249	0.0017*	0.0401	0.9476	0.6542	

*The Bonferroni adjustment for multiple comparisons was, used and significance was declared if p-value was ≤ 0.0033

The p-values for pairwise comparisons are below diagonal, and estimates with standard errors are above diagonal.

Table A2 (continued)

Trait	Haplotypes	h1	h2	h7	h8	h9	h12
10:0	h1		-0.151 0.053	-0.077 0.045	-0.013 0.046	-0.003 0.042	-0.022 0.044
	h2	0.0044		0.074 0.048	0.138 0.049	0.148* 0.044	0.129 0.049
	h7	0.086	0.1277		0.064 0.036	0.074 0.031	0.056 0.037
	h8	0.7808	0.0054	0.0722		0.010 0.034	-0.009 0.041
	h9	0.9432	0.0008*	0.0151	0.7726		-0.019 0.034
	h12	0.6241	0.0091	0.1325	0.8306	0.583	

Trait	Haplotypes	h1	h2	h7	h8	h9	h12
13:0	h1		-0.015* 0.005	-0.005 0.004	-0.005 0.004	-0.007 0.004	0.001 0.004
	h2	0.0024*		0.009 0.004	0.009 0.004	0.007 0.004	0.016* 0.004
	h7	0.211	0.0318		-0.000 0.003	-0.002 0.003	0.006 0.003
	h8	0.2079	0.0394	0.9578		-0.002 0.003	0.006 0.004
	h9	0.0544	0.0682	0.4276	0.5129		0.009 0.003
	h12	0.7587	0.0004*	0.059	0.0787	0.0055	

Trait	Haplotypes	h1	h2	h7	h8	h9	h12
14:1	h1		0.006 0.024	0.043 0.021	0.035 0.021	-0.030 0.019	0.072* 0.020
	h2	0.8169		0.037 0.022	0.030 0.023	-0.035 0.020	0.066* 0.023
	h7	0.038	0.0904		-0.007 0.016	-0.073* 0.014	0.029 0.017
	h8	0.0959	0.1872	0.6524		-0.065* 0.016	0.037 0.019
	h9	0.1213	0.077	<.0001*	<.0001*		0.102* 0.016
	h12	0.0004*	0.0033*	0.0837	0.0513	<.0001*	

Table A2 (continued)

Trait	Haplotypes	h1	h2	h7	h8	h9	h12
17:0	**h1**		0.0029 0.0056	0.0031 0.0047	-0.0048 0.0049	-0.0099 0.0044	0.0057 0.0047
	h2	0.5985		0.0002 0.0051	-0.0077 0.0052	-0.0128 0.0046	0.0028 0.0052
	h7	0.5108	0.9705		-0.0079 0.0038	-0.0130* 0.0032	0.0026 0.0039
	h8	0.3281	0.1401	0.0365		-0.0051 0.0036	0.0105 0.0043
	h9	0.0252	0.0056	<.0001*	0.1549		0.0156* 0.0036
	h12	0.2183	0.5893	0.5018	0.0149	<.0001*	

Trait	Haplotypes	h1	h2	h7	h8	h9	h12
C14 Index	**h1**		0.0020 0.0021	0.0046 0.0017	0.0032 0.0018	-0.0025 0.0016	0.0057 0.0017
	h2	0.3366		0.0026 0.0019	0.0013 0.0019	-0.0045 0.0017	0.0037 0.0019
	h7	0.0094	0.1691		-0.0013 0.0014	-0.0071 0.0012	0.0012 0.0014
	h8	0.0725	0.5123	0.3435		-0.0058 0.0013	0.0025 0.0016
	h9	0.1215	0.0085	<.0001*	<.0001*		0.0082 0.0013
	h12	0.0009*	0.0518	0.4194	0.1197	<.0001*	

Table A3 Pairwise comparisons of AGPAT1 haplotype effects on milk fatty acid composition

Trait	Haplotypes	h2	h4	h6	h9	h10
PUFA	**h2**		0.018 0.060	0.035 0.041	-0.098 0.053	-0.123* 0.036
	h4	0.76		0.016 0.067	-0.116 0.076	-0.141 0.065
	h6	0.4007	0.8056		-0.133 0.063	-0.157* 0.049
	h9	0.0681	0.1276	0.0362		-0.025 0.062
	h10	0.0008*	0.0295	0.0013*	0.689	

Trait	Haplotypes	h2	h4	h6	h9	h10
6:0	**h2**		0.018 0.024	0.006 0.017	0.057 0.021	0.045* 0.015
	h4	0.4629		-0.011 0.027	0.039 0.031	0.027 0.026
	h6	0.7055	0.6689		0.050 0.025	0.038 0.019
	h9	0.008	0.2023	0.0452		-0.012 0.025
	h10	0.0023*	0.3021	0.0487	0.6272	

Trait	Haplotypes	h2	h4	h6	h9	h10
8:0	**h2**		0.009 0.017	0.005 0.012	0.031 0.015	0.027 0.010
	h4	0.5882		-0.004 0.019	0.022 0.022	0.017 0.019
	h6	0.6541	0.8337		0.026 0.018	0.021 0.014
	h9	0.0408	0.3136	0.1496		-0.005 0.018
	h10	0.0112	0.3549	0.1283	0.7869	

*The Bonferroni adjustment for multiple comparisons was used, and significance was declared if p-value was ≤ 0.005

The p-values for pairwise comparisons are below diagonal, and estimates with standard errors are above diagonal.

Table A3 (continued)

Trait	Haplotypes	h2	h4	h6	h9	h10
14:1	**h2**		0.017 0.022	0.005 0.015	-0.067* 0.020	0.019 0.014
	h4	0.4455		-0.012 0.025	-0.084* 0.028	0.001 0.024
	h6	0.7273	0.6383		-0.073* 0.023	0.013 0.018
	h9	0.0007*	0.0029*	0.002*		0.086* 0.023
	h10	0.1727	0.9533	0.4689	0.0002*	

Trait	Haplotypes	h2	h4	h6	h9	h10
15:0	**h2**		0.031 0.018	-0.010 0.012	-0.012 0.016	-0.020 0.011
	h4	0.0812		-0.041 0.020	-0.043 0.023	-0.051 0.019
	h6	0.4262	0.0398		-0.002 0.019	-0.010 0.015
	h9	0.4629	0.0591	0.9181		-0.008 0.019
	h10	0.0742	0.0085	0.511	0.6831	

Trait	Haplotypes	h2	h4	h6	h9	h10
17:0	**h2**		0.0090 0.0049	-0.0002 0.0034	0.0038 0.0044	-0.0072 0.0030
	h4	0.0676		-0.0092 0.0055	-0.0052 0.0063	-0.016* 0.0053
	h6	0.9552	0.0942		0.0040 0.0052	-0.0070 0.0040
	h9	0.3881	0.4055	0.4424		-0.0111 0.0051
	h10	0.016	0.0023*	0.0795	0.0316	

Table A3 (continued)

Trait	Haplotypes	h2	h4	h6	h9	h10
18:2	**h2**		-0.029 0.043	0.035 0.030	-0.038 0.038	-0.082* 0.026
	h4	0.4942		0.064 0.048	-0.009 0.054	-0.053 0.046
	h6	0.2407	0.1803		-0.073 0.045	-0.117* 0.035
	h9	0.316	0.8701	0.1054		-0.044 0.044
	h10	0.0017*	0.2544	0.0008*	0.3229	

Trait	Haplotypes	h2	h4	h6	h9	h10
CLA	**h2**		0.020 0.020	0.003 0.014	-0.044 0.018	-0.035* 0.012
	h4	0.3374		-0.016 0.023	-0.064 0.026	-0.055 0.022
	h6	0.8098	0.4745		-0.047 0.021	-0.039 0.016
	h9	0.0147	0.0137	0.0259		0.009 0.021
	h10	0.0044*	0.0129	0.0192	0.6753	

Table A4 Pairwise comparisons of LPIN1 haplotype effects on milk fatty acid composition

Trait	Haplotype	h1	h4	h13	h18	h22	h24	h26
14:1	**h1**		-0.025 0.019	-0.036 0.021	-0.037 0.019	-0.085* 0.023	-0.004 0.020	-0.007 0.018
	h4	0.1795		-0.011 0.022	-0.012 0.019	-0.060 0.023	0.021 0.021	0.018 0.018
	h13	0.084	0.6162		-0.001 0.022	-0.049 0.024	0.032 0.022	0.029 0.021
	h18	0.0556	0.5309	0.978		-0.048 0.023	0.033 0.021	0.030 0.018
	h22	0.0002*	0.01	0.041	0.0332		0.081* 0.024	0.078* 0.022
	h24	0.8355	0.3165	0.152	0.121	0.001*		-0.003 0.020
	h26	0.6989	0.3086	0.1574	0.0939	0.0003*	0.8891	

Trait	Haplotype	h1	h4	h13	h18	h22	h24	h26
C14 Index	**h1**		-0.0020 0.0016	-0.0027 0.0018	-0.0032 0.0016	-0.0073* 0.0020	-0.0000 0.0017	-0.0005 0.0015
	h4	0.2202		-0.0008 0.0019	-0.0012 0.0016	-0.0053 0.0020	0.0020 0.0018	0.0014 0.0015
	h13	0.1263	0.6822		-0.0005 0.0019	-0.0045 0.0020	0.0028 0.0019	0.0022 0.0018
	h18	0.0527	0.4396	0.8091		-0.0041 0.0019	0.0032 0.0018	0.0027 0.0015
	h22	0.0002*	0.0074	0.0256	0.0343		0.0073* 0.0021	0.0067* 0.0018
	h24	0.9866	0.2647	0.1468	0.0735	0.0005*		-0.0006 0.0017
	h26	0.73	0.3452	0.2115	0.0804	0.0003*	0.7392	

*The Bonferroni adjustment for multiple comparisons was used, and significance was declared if p-value was ≤ 0.005

The p-values for pairwise comparisons are below diagonal, and estimates with standard errors are above diagonal.

APPENDIX B. SUPPLIMENTARY INFORMATION FOR CHAPTER 3

Table B1 Pairwise comparisons of SLC27A6 haplotype effects on milk fatty acid composition

Trait	Haplotypes	h1	h5	h6	h9	h10
Milk fat	h1		0.048 0.033	-0.052 0.036	-0.119 0.046	0.032 0.028
	h5	0.1445		-0.100 0.043	-0.167* 0.051	-0.016 0.038
	h6	0.1473	0.0215		-0.067 0.056	0.083 0.040
	h9	0.0109	0.001*	0.2292		0.151* 0.049
	h10	0.2569	0.6651	0.0381	0.0021*	

Trait	Haplotype	h1	h5	h6	h9	h10
AI	h1		0.046 0.020	-0.052 0.022	-0.029 0.028	-0.004 0.017
	h5	0.0221		-0.098* 0.026	-0.075 0.031	-0.050 0.023
	h6	0.0168	0.0002*		0.022 0.034	0.047 0.025
	h9	0.2949	0.0138	0.5101		0.025 0.030
	h10	0.7929	0.0284	0.0538	0.4002	

Trait	Haplotype	h1	h5	h6	h9	h10
SFA	h1		0.371 0.213	-0.633 0.229	-0.375 0.299	-0.087 0.181
	h5	0.0813		-1.004* 0.279	-0.746 0.325	-0.458 0.244
	h6	0.0058	0.0003*		0.258 0.359	0.546 0.259
	h9	0.2103	0.0221	0.4726		0.288 0.315
	h10	0.63	0.0602	0.0355	0.3615	

*The Bonferroni adjustment for multiple comparisons was used, and significance was declared if p-value was ≤ 0.005

The p-values for pairwise comparisons are below diagonal, and estimates with standard errors are above diagonal.

Table B1 (continued)

Trait	Haplotype	h1	h5	h6	h9	h10
UFA	h1		-0.371 0.213	0.633 0.229	0.375 0.299	0.087 0.181
	h5	0.0813		1.004* 0.279	0.746 0.325	0.458 0.244
	h6	0.0058	0.0003*		-0.258 0.359	-0.546 0.259
	h9	0.2103	0.0221	0.4726		-0.288 0.315
	h10	0.63	0.0602	0.0355	0.3615	

Trait	Haplotype	h1	h5	h6	h9	h10
MUFA	h1		-0.376 0.193	0.536 0.208	0.228 0.271	0.042 0.164
	h5	0.051		0.912* 0.253	0.604 0.294	0.418 0.221
	h6	0.01	0.0003*		-0.308 0.326	-0.494 0.235
	h9	0.4005	0.0404	0.3438		-0.186 0.285
	h10	0.799	0.0584	0.0359	0.5152	

Trait	Haplotype	h1	h5	h6	h9	h10
SFA/UFA	h1		0.027 0.016	-0.044 0.017	-0.033 0.022	-0.007 0.014
	h5	0.086		-0.072* 0.021	-0.060 0.024	-0.034 0.018
	h6	0.0101	0.0006*		0.011 0.027	0.037 0.019
	h9	0.1433	0.0137	0.6726		0.026 0.024
	h10	0.6122	0.0607	0.0547	0.2718	

Table B1 (continued)

Trait	Haplotype	h1	h5	h6	h9	h10
10:0	h1		0.068 0.031	-0.097* 0.034	0.026 0.044	0.009 0.027
	h5	0.0314		-0.165* 0.041	-0.042 0.048	-0.059 0.036
	h6	0.0042*	<.0001*		0.123 0.053	0.106 0.038
	h9	0.5602	0.3814	0.0209		-0.017 0.047
	h10	0.7424	0.1023	0.0059	0.7167	

Trait	Haplotype	h1	h5	h6	h9	h10
12:0	h1		0.079 0.035	-0.108* 0.038	0.050 0.050	0.000 0.030
	h5	0.0261		-0.187* 0.046	-0.029 0.054	-0.079 0.041
	h6	0.0048*	<.0001*		0.158 0.060	0.108 0.043
	h9	0.317	0.5882	0.0085		-0.050 0.052
	h10	0.9883	0.0511	0.0132	0.3396	

Trait	Haplotype	h1	h5	h6	h9	h10
14:0	h1		0.180 0.069	-0.145 0.075	0.099 0.097	-0.004 0.059
	h5	0.0096		-0.324* 0.091	-0.081 0.105	-0.184 0.079
	h6	0.0539	0.0004*		0.243 0.117	0.141 0.085
	h9	0.3068	0.4413	0.0375		-0.103 0.102
	h10	0.9456	0.021	0.0987	0.3154	

Table B1 (continued)

Trait	Haplotype	h1	h5	h6	h9	h10
16:0	h1		0.137 0.124	-0.120 0.134	-0.496* 0.175	-0.085 0.106
	h5	0.2699		-0.257 0.163	-0.633* 0.190	-0.223 0.142
	h6	0.3706	0.1151		-0.376 0.210	0.034 0.151
	h9	0.0047*	0.0009*	0.0736		0.410 0.184
	h10	0.4189	0.1178	0.8203	0.026	

Table B2 Pairwise comparisons of FABP3 haplotype effects on milk fatty acid composition

Trait	Haplotype	h1	h2	h3
15:0	h1		-0.050* 0.016	-0.021 0.011
	h2	0.0027*		0.028 0.019
	h3	0.0464	0.1271	

Trait	Haplotype	h1	h2	h3
Elongation index	h1		0.008* 0.003	0.003 0.002
	h2	0.0051*		-0.004 0.003
	h3	0.0634	0.1492	

*The Bonferroni adjustment for multiple comparisons was used, and significance was declared if p-value was ≤ 0.0167

The p-values for pairwise comparisons are below diagonal, and estimates with standard errors are above diagonal.

Table B3 Pairwise comparisons of FABP4 haplotype effects on milk fatty acid composition

Trait	Haplotype	h1	h4	h5
SFA	h1		-0.057 0.176	-0.569* 0.182
	h4	0.7447		-0.512* 0.199
	h5	0.0018*	0.0101*	

Trait	Haplotype	h1	h4	h5
UFA	h1		0.057 0.176	0.569* 0.182
	h4	0.7447		0.512* 0.199
	h5	0.0018*	0.0101*	

Trait	Haplotype	h1	h4	h5
MUFA	h1		0.081 0.159	0.475* 0.164
	h4	0.6107		0.394 0.180
	h5	0.0039*	0.0285	

Trait	Haplotype	h1	h4	h5
PUFA	h1		-0.016 0.033	0.105* 0.034
	h4	0.6297		0.122* 0.038
	h5	0.0022*	0.0012*	

Trait	Haplotype	h1	h4	h5
SFA/UFA	h1		-0.003 0.013	-0.040* 0.014
	h4	0.8244		-0.037* 0.015
	h5	0.0034*	0.0129*	

*The Bonferroni adjustment for multiple comparisons was used, and significance was declared if p-value was ≤ 0.0167

The p-values for pairwise comparisons are below diagonal, and estimates with standard errors are above diagonal.

Table B3 (continued)

Trait	Haplotype	h1	h4	h5
18:2	h1		-0.014 0.024	0.067* 0.025
	h4	0.5692		0.080* 0.027
	h5	0.0068*	0.0029*	

Trait	Haplotype	h1	h4	h5
CLA	h1		0.0067 0.0111	0.0353* 0.0115
	h4	0.544		0.0285 0.0126
	h5	0.0022*	0.0234	

Trait	Haplotype	h1	h4	h5
Index 18	h1		-0.0002 0.0019	0.0051* 0.0020
	h4	0.9325		0.0053* 0.0021
	h5	0.0088*	0.0134*	

APPENDIX C. SUPPLIMENTARY INFORMATION FOR CHAPTER 4

Table C1 Pairwise comparisons of SREBP1 haplotype effects on milk fatty acid composition

Trait	Haplotype	h1	h4	h5
14:0	h1		0.076 0.059	0.226* 0.057
	h4	0.2013		0.150 0.070
	h5	<.0001*	0.0314	

Trait	Haplotype	h1	h4	h5
14:1	h1		-0.0360* 0.0129	0.0069 0.0123
	h4	0.0055*		0.0429* 0.0151
	h5	0.5743	0.0046*	

Trait	Haplotype	h1	h4	h5
Index 14	h1		-0.0033* 0.0011	-0.0010 0.0011
	h4	0.0031*		0.0023 0.0013
	h5	0.351	0.0757	

*The Bonferroni adjustment for multiple comparisons was used, and significance was declared if p-value was ≤ 0.0167

The p-values for pairwise comparisons are below diagonal, and estimates with standard errors are above diagonal.

Table C2 Pairwise comparisons of Insig1 haplotype effects on milk fatty acid composition

Trait	Haplotype	h3	h7	h23
MUFA	**h3**		-0.015 0.165	-0.263 0.154
	h7	0.9269		-0.248 0.160
	h23	0.089	0.1213	

Trait	Haplotype	h3	h7	h23
PUFA	**h3**		0.071 0.035	0.099* 0.032
	h7	0.0413		0.028 0.034
	h23	0.0024*	0.4054	

Trait	Haplotype	h3	h7	h23
16:0	h3		0.028 0.108	0.040 0.101
	h7	0.7921		0.011 0.105
	h23	0.6946	0.9141	

Trait	Haplotype	h3	h7	h23
18:2	**h3**		0.047 0.024	0.083* 0.023
	h7	0.0571		0.036 0.024
	h23	0.0003*	0.1263	

*The Bonferroni adjustment for multiple comparisons was used, and significance was declared if p-value was ≤ 0.0167

The p-values for pairwise comparisons are below diagonal, and estimates with standard errors are above diagonal.